MOTOR NEURONE AND MUSCLE NUCLEAR CHANGES IN DEVELOPMENT AND DISEASE

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S U M M A R Y

The primary gene defects of hereditary neuromuscular disorders have not, as yet, been determined. Research has contributed much to our understanding of many aspects of these diseases, but since this has been directed towards the secondary phenomena, the exact role of the nucleus in the pathogenesis of these genetic disorders remains unclear. It was felt that a study of the motor neurone and muscle nucleus in development and disease might reveal the extent of its participation in some of these disease processes.

1. A quantitative histochemical and histometric study of the developing anterior horn cell nucleus during fetal life was undertaken. The result suggested that the 12th to 14th week period is critical for the differentiation of the spinal motor neurone.

2. In contrast to this, a similar study of the developing muscle cell nuclei revealed no major changes in muscle nuclear size or composition. During normal post-natal

muscle growth an increase in the number of nuclei per muscle fibre seems to be responsible for the maintenance of a constant nucleo-cytoplasmic ratio.

3. The nuclear size of multinucleated myoblasts arising from both normal and dystrophic muscle in tissue culture was measured. Myoblasts from dystrophic muscle exhibited larger nuclei than those from normal muscle.

4. An increase in muscle nuclear size was also detected in a number of muscle samples from male fetuses at risk for Duchenne muscular dystrophy. Both of these findings suggest possible changes in the nucleo-cytoplasmic relationship.

5. In an attempt to elucidate the extent of the participation of the muscle nucleus in the pathogenic process of a number of characteristic neuromuscular disorders, the nuclear size and/or the number of nuclei per cross-sectioned muscle fibre, were estimated. An increased nuclear size was observed in Duchenne muscular dystrophy and an increase in the number of nuclei was detected in chronic neurogenic atrophies, in diabetic neuropathy and in myotonic dystrophy. Such a finding would seem to indicate a fundamental difference in the response of the muscle fibre nucleus to the neurogenic and myopathic processes.

6. In addition, the growth of the human cervical vertebral canal and spinal cord during normal fetal development was studied. The results suggested that these two parts of the

developing body exhibit the same developmental pattern. The rate of this parallel growth is lower than that of the body as a whole, but similar to that reported for the brain.

In conclusion, although the techniques used did not reveal a direct relationship in the developmental pattern of anterior horn cell nuclei and myonuclei, they did provide normal values for comparison with data obtained from diseased neural and muscle tissue. The finding that in Duchenne muscular dystrophy (from tissue culture, fetuses at risk and juvenile biopsies) the muscle nuclei are enlarged, points to an underlying difference from various neuropathies in which the nuclei are increased in number.

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C O N T E N T S

INTRODUCTION

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Neuromuscular diseases have classically been divided into those of neurogenic and those of myopathic origin. Although the exact pathogenesis of these diseases remains unknown, in the former there is little doubt about the primary involvement of the neural tissue, whereas the "myopathic" origin of some muscular dystrophies has recently been strongly challenged.

Doubt was first cast upon this clinically accepted division of neuromuscular diseases by the report of Kugelberg and Welander (1956), who described patients with spinal muscular atrophy closely resembling muscular dystrophy. In addition, Zellweger et al. (1965) and Zellweger and Hanson (1967)

reported electroencephalographic abnormalities and a degree of mental retardation in a proportion of patients with Duchenne muscular dystrophy (DMD).

The first direct, but most controversial evidence, that the muscular dystrophies may be due to a primary neuronal abnormality came from electrophysiological studies. McComas et al. (1971) reported a reduction in the number of motor units in various types of muscular dystrophy, and interpreted these results as suggesting that muscular dystrophy is associated with "sick motor neurones". This hypothesis (and McComas' technique in particular) has since been seriously criticised (Scarpalezos and Panayiotopoulos 1973, Panayiotopoulos et al. 1974, Thomson et al. 1974, Ballantyne and Hanson 1974 a, b). However, it has provided a stimulus for further research into the pathogenesis of neuromuscular disorders.

A tremendous amount of data from various disciplines have appeared both in support of, and refuting the "neurogenic hypothesis". As well as neurophysiology, evidence has come from animal pathology, transplantation and cross-innervation experiments and tissue culture studies.

From the point of view of animal pathology, changes in the motor nerves and anterior roots in mice with "muscular dystrophy" have been reported (Harris et al., 1972, Bradley and Jekinson 1973), but the number and structure of the anterior horn cells of the spinal cord in both human and murine muscular dystrophy are normal (Papapetropoulos and Bradley 1972,

Tomlinson et al. 1974). Lin and Hartman (1971) reported a significant decrease in the amount of guanine in the neurone cytoplasmic RNA in drug induced dystrophy in rats. In humans Rosman and Kakulas (1966) have observed brain abnormalities (pachygyria, heterotopias etc.) in children with DMD.

Transplantation experiments have showed that dystrophic muscle transplanted into normal mice survives well (Laird and Timmer 1966, Salafsky 1971, Hironaka and Miyata 1973), but the interpretation of transplantation experiments is still equivocal.

The recent development of tissue culture techniques has offered a fascinating approach to the study of neuromuscular diseases. A lack of differentiation into fibre types in the absence of innervation has been reported by Askanas et al., (1972) and Gallup et al., (1972). Gallup and Dubowitz (1973) provided further evidence for the importance of a neuronal factor; in in vitro studies they reported that although normal mouse spinal cord supports the regeneration of both dystrophic and normal muscle, the dystrophic spinal cord does not seem to do this. A number of studies (Skeate et al. 1969, Bishop et al. 1971, Gallup et al. 1972) have suggested that there are no marked differences in the growth and differentiation between human, normal and dystrophic muscle. However, a delay in the myotube maturation in human muscular dystrophy has been reported by Bateson et al. (1972). Several workers (Hamburgh et al. 1973, Paul and Powell 1974) have reported results contradictory to those of Gallup and Dubowitz.

The role of the muscle nucleus in the dystrophic process has been investigated by Peterson's (1974) chimaera formation technique. He found that although some fibres had only dystrophic nuclei there was little or no pathological degeneration of the fibres. On the contrary, muscle containing no dystrophic nuclei sometimes showed pathological changes. Based on these observations Peterson suggested that mouse muscle degeneration is caused by some abnormality of extramuscular origin.

The evidence for a neurogenic component in muscular dystrophy is still conflicting and as Emery and Gosden (1974) noted, this component cannot be simply a defect in the anterior horn cells since the clinical, the pathological, the electrophysiological and other laboratory findings are quite different from those in spinal muscular atrophy.

The anatomical and physiological complexity of the nervous system, as well as the fact that until recently the detailed anatomy of the motor unit had received little study, would, to some extent, explain the existing conflict about the involvement of the various parts of the motor unit in the pathologic process of the so-called myopathies. Functionally, one of the most complex parts of the nervous system is the anterior horn cell of the spinal cord which, with its axon, is usually referred to as the "final common pathway". The concept of this pathway is quite fundamental to an understanding of the function of the nervous system, since all motor impulses from all levels of the motor system converge, directly or indirectly,

on the motor neurones. This indicates the complexity and importance of motor neurone interactions. The summation of impulses arriving at those cells is extremely complex and variable; the final result of this complexity can be no more than pattern of impulses along the axons (temporal patterns in individual neurones and spatial patterns in groups of them) (Elliott 1969).

The anterior horn cell is one of the largest cells of the human body with enormous bushes of dendrites (for receiving stimuli), but only one axon (for transmission) and each neurone may innervate up to 200 muscle fibres. They are remarkably few in number (it has been estimated that the number of motor neurones in the human spinal cord is only 80-160.000) in comparison with the hundreds of millions estimated for various parts of the brain (basal ganglia, cerebellum etc.).

Thus, it can be appreciated why the motor neurone is the most vulnerable part of the motor system. Relatively minor lesions result in the loss of a high proportion of motor neurones; the end result of such a loss is magnified, since the motor neurone represents a "final common pathway for which nothing can be substituted" (Elliott 1969).

The other component of the motor unit, the muscle, has been the focus of much work in the last 15-20 years and it is only recently that we have been able to build up some sort of general picture of the structure and the function of the muscle. Advances in enzyme histochemistry and the application of new tissue culture

techniques haveallowed a better understanding of the way the muscle acts under normal and abnormal conditions, as well as the existing interaction between the peripheral nervous system and the muscle. The arrangement and function of the fibrillar proteins which form the contractile part of the muscle as well as the features of the T-system and the sarcoplasmic reticulum have been elucidated by electronmicroscopy. In addition, the electronmicroscopy has provided valuable information about the ultrastructural characteristics of the various fibre types and the intrafusal fibres of the muscle spindle. Finally, the identification of the satellite cells by Mauro (1961) has thrown a new light in the phenomenon of muscle regeneration. The histochemical pattern of the muscle has been extensively studied and the enzymatic activity of the muscle fibre under normal and pathological conditions is well established. (Beckett and Bourne 1972, Dubowitz and Brooke 1973, Dubowitz 1974).

Many authors (Campa and Engel 1970 a, b, Odutola 1972, Penny et al. 1975) have attempted to establish histochemical differences in the anterior horn cells of the spinal cord (especially in the alpha motor neurones). Although such differences have been reported, the similarity of phosphorylase and succinic dehydrogenase (SDH) activity between motor neurones innervating fast twitch muscle and those innervating slow ones contrasts with the marked histochemical differences observed between these two main muscle fibre types. Thus, it would seem that there is no simple correlation between the functional properties of the two types of alpha

motor neurone with those of the muscle fibres they innervate (Wuerker et al. 1965, Burke 1967, Campa and Engel 1971).

Although much research has been done on the functional properties of the cytoplasm of both the motor neurones and muscle fibres, scant attention has been paid to the nucleus of these cells in spite of the well recognised integral role of the nucleus in cell function.

The age of onset of the neuromuscular diseases is very variable, but for many of them (Werdnig-Hoffmann, Duchenne muscular dystrophy) it has been suggested that the pathological process may be evident in early fetal life (Toop and Emery 1974, Webb 1974, Walton 1974, Vassilopoulos and Emery, 1976). Since these diseases are genetic in origin, involvement of the nucleus to a lesser or greater extent in the pathologic process is expected.

It is now well established that the genes which transmit hereditary information are composed of DNA. Apart from the DNA the nucleus also contains two main protein components, namely histones and acidic (non-histone) nuclear proteins. These proteins have been implicated in the mechanisms operating in transcription and translation of genetic information.

The complete development of the motor unit depends on the differentiation of the lower motor neurone. This process of differentiation which results in a highly specialised anterior horn cell, involves selective transcription of specific regions of the DNA to produce the RNA populations characteristic and necessary to these cells. This selective and partial expression of the genome occurs

in spite of the presence, in most diploid cells, of a complete DNA complement sufficient to specify the formation of an entire organism. It is now clear that individual cells suppress the transcription of most of their DNA while they selectively activate a small number of genes necessary for the synthesis assembly, processing and post synthetic modification of enzymes and structural proteins characteristic of the cell type. In the course of embryonic development and ageing, different sets of genes are activated or repressed in response to programmed signals from the nucleus, the cytoplasm, the cell membrane and finally the environment. This response is mediated through the action of the nuclear proteins which influence the structure of the genetic material so transmitting physiological control signals for gene activation or repression. The main nuclear protein fractions include the histones which are the basic suppressive component of the chromatin and the acidic nuclear proteins (at least some of them) involved in selective derepression of the genetic material. (Kischer and Hnilica 1967, Hnilica 1967, Teng et al. 1971, Vidali et al. 1973, Olson and Busch 1974, Stein et al. 1975, Allfrey 1975, LeSturgeon et al 1975).

This study is concerned with anterior horn cell differentiation and is based on the theory that differentiation is the result of programmed sequential activation, inactivation, or other quantitative regulations of specific genes. It is felt that efforts to elucidate the defective genetic mechanism operating in the neuromuscular diseases may be facilitated by studies on differentiating anterior

horn cells.

At the present time we are unable to detect the particular single gene defect in various diseases and information can only be obtained by detection of the resulting defect in the metabolic pathway. The characteristic features that are observed in any disorder often represent the end results of complex phenomena which involve interactions at many different levels of the biochemical and physiological organisation. It is only in a few conditions that it has been possible, so far, to piece together some details of the causal sequence of events. Since a direct approach at a molecular level is at present difficult, information must be obtained indirectly by considering nuclear and nucleolar size which reflect the level of the cell activity. The nucleolus in particular plays a very important role in protein, particularly RNA, synthesis and its size has been considered as an index of the synthetic activity of the cell (Stowell 1948, Kleinfeld 1957, Busch et al. 1963, Giese 1968, Smetana and Busch 1974). Changes in nuclear and nucleolar size in various tissues have been observed in a number of diseases as well as in animals under experimental conditions. In the human anterior horn cells, changes in nuclear and nucleolar volume have been observed and a correlation between nuclear and nucleolar size and RNA content has been reported in "motor neurone disease" (Mann and Yates 1974). The authors suggested that the nucleus is the initial site of action of the causative "pathogen" in this disease. In the muscle, alterations in nuclear size have been correlated with various functional states (muscle

contraction, altered ionic environment etc.) (Franke and Schinko 1969, Davies and Spencer 1962). Changes in the size of the nucleus could be considered of importance since they probably reflect modification of nucleo-cytoplasmic interactions. Further information concerning these interactions has been obtained from nuclear transplantation experiments in amphibia (Gurdon 1968; 1970).

The present study set out to characterise the histochemical pattern of the anterior horn cell nucleus as well as the muscle nucleus during normal human fetal development. Since nuclear and nucleolar size are related to the activity of a cell, their size was measured during the development of both the anterior horn cell and the muscle fibre; in addition, the size and the number of the muscle fibre nuclei under various pathological conditions were estimated, in the hope that the information gained would be helpful in understanding more of the pathogenic processes of various neuromuscular disorders. Finally, since certain hereditary conditions seem to modify the relationship between spinal cord and vertebral canal, the growth of these structures was studied in order to elucidate their normal developmental pattern during fetal development.

CHAPTER 1

Motor neurone nuclear changes during fetal development

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CHAPTER ONE

I. Introduction

The neurones of all the cells in the human body are the most difficult to study under in vivo conditions. This problem is magnified when the differentiation and the development of the neurones is studied. A major difficulty in obtaining a unified picture of neuronal development is the diversity of the published data regarding species, ages, tissue preparation and type of cell studied.

The neurone can be regarded as a "dual" entity with

conductive and trophic capacities interrelated in diverse ways. The evolvement of this duality may be followed during fetal development. The cell surface expands with growth allowing increased communication with other cells; at the same time the organelles associated with intracellular metabolism are elaborated. Regardless the dissimilarity in appearance of many mature neurones, there is a common intracellular developmental sequence starting from the primitive pre-differentiated state.

LaVelle and LaVelle (1970) distinguished three periods of nuclear and cytoplasmic growth during vertebrate neuronal development. The first period involves the emergence of a recognisable neuroblast from the primitive neuroepithelium, its migration to its new locus and the extension of its primary neurite. All the neuroblast still appear rather similar at this stage. The second period begins after the neuroblast has reached its definite site and has perhaps established certain basic connections with its target field. This period is marked by accelerated somal and nuclear growth and by differentiation of organelles to such an extent that the various neuronal cell types are clearly recognisable. The third period of neuronal growth comprises the completion of the dendritic tree and the achievement of the final somal and nuclear size as the cell becomes trophically mature. A further increase in volume may occur in the late phase of

this period the extent of which depends on the animal.

From the onset of cytological differentiation until neuronal maturity, the nucleus and the nucleolus of a neurone pass through a definite sequence of changes in volume, staining density, position and shape. In the first period of neuronal development, the nuclear chromatin becomes more dispersed and stains less densely. The nucleus is eccentric and ellipsoid. In the second period the nucleus becomes central, almost spherical and the nuclear basophilia decreases. This stage is characterised by a pronounced increase in the nuclear volume with a corresponding increase in the total quantity of nuclear proteins (LaVelle and LaVelle 1970). In the third period the nuclear volume continues to increase to a final value, which is not fixed, but it is probably influenced by factors extrinsic to the neurone itself.

The nucleolus, like the nucleus, increases in size mainly during the second period and this enlargement is accompanied by a concomitant increase in cytoplasmic Nissl substance (LaVelle 1956, LaVelle and LaVelle 1970). The number of nucleoli and their staining properties during development have been correlated to the functional activity of the cell. (LaVelle and LaVelle 1970, Zilles et al. 1976). The final size of the nucleolus is directly correlated with the final size of the neurone body and the total amount of Nissl substance produced. (LaVelle 1956, Edström and Eichner 1958).

It is now clear that the ability of a neurone to transmit impulses and the ability to grow and to maintain itself as a given neuronal type requires a certain structural and functional organization which is the result of differentiation. The differentiation involves spatio-temporal selective transcription of specific regions of DNA and inactivation or suppression of undesirable segments of the originally multipotent DNA complex of the primitive neuroepithelium. These two aspects of transcriptional control (suppression of the template activity of most of the DNA and activation of RNA synthesis at particular genetic loci) require the participation of proteins associated with the chromatin complex. These proteins influence the structure of the genetic material modifying its interactions with RNA polymerases and in addition they transmit physiological control signals for gene activation or repression in response to hormones, cyclic nucleotides and other types of stimuli.

The main protein fractions concerned with the structure and function of chromatin include histones (which suppress the genome) and the acidic nuclear proteins which play a positive role in the selective aspects of genetic control. Among the many possible mechanisms, histones are suspected to play the role of genetic repressors and this rather old hypothesis (Stedman and Stedman 1950) has received experimental support from studies on inhibition of RNA synthesis in isolated cell nuclei (Allfrey et al. 1963) or in chromatin

fractions (Huang and Bonner 1962). Further studies however, made it clear that the role of histones is largely structural. The histone metabolism, in the metaphase nucleus, mainly involves postsynthetic modifications and such alterations seem to be involved in the control of chromatin structure. Histone modification reactions may influence the transcriptional process by altering the physical state of the DNA template (Allfrey 1971, MacGillivray and Rickwood 1974), but histone modifications themselves are not sufficient for the iniation of RNA synthesis at any gene loci (Ono et al. 1969, Allfrey 1971). The possible role of the various histone fractions in embryogenesis has been investigated (Agrell and Christensson 1965, Hnilica 1967, Auer and Zetterberg 1972, Piha and Jokela 1972), but still little is known about the role of the arginine- and lysine-rich fractions in the process of cell differentiation. In any case, it now seems clear that histones are regulatory molecules involved in the control of gene transcription, but their lack of specificity (because of their limited number and complexity) which precludes their ability to recognise and influence particular genes, suggesting that if they regulate transcription they do it in a nonspecific sense.

The other major nuclear component, the acidic nuclear proteins are synthesized, like the histones, not in the nucleus, but in the cytoplasm and are then transported into the nucleus to become associated with DNA. However, although histones

and DNA appear to constitute permanent components of the genome, the acidic nuclear proteins are, at least partly, in a state of dynamic flux. (Stein et al. 1975). It is believed that only a proportion are involved in the control of transcription; many are concerned with chromosome structure and mobility, transport of gene products and enzymatic modifications of the nuclear proteins. However, there is good evidence that some of the acidic nuclear proteins influence the rate and specificity of RNA synthesis and transmit information from the cytoplasm, the cell membranes and other parts of the nucleus to genetic loci. Evidence that a proportion of the acidic nuclear proteins are involved in transcriptional control are summarised as follows:

1. The nature and the amount of the acidic nuclear proteins change during embryogenesis (Hill et al. 1971, Seale and Aronson 1973, Spelsberg et al. 1973).

2. There is a decrease of the heterogeneity of the acidic nuclear proteins when proliferating cells differentiate into non-dividing forms and an increased heterogeneity is associated with cellular proliferation (Hnilica 1967).

3. Their distribution varies in different somatic tissues (Teng et al. 1971, Chytil and Spelsberg 1971, Wang 1971, Zardi et al. 1973), as would be expected if acidic nuclear proteins are involved in gene activity.

4. The activated "puffing" regions of insect chromosomes accumulate specific nuclear proteins after stimulation (Helmsing and Berendes 1971) and protein phosphorylation is most pronounced over these "puffing" regions.

5. An increase in the synthesis of acidic nuclear proteins is observed at the early stages of cell proliferation in salivary gland cells stimulated by isoprenaline (Stein and Baserga 1970) and in lymphocytes stimulated by phytohemagglutinin (Levy et al. 1973).

6. Acidic nuclear proteins can directly influence the rate of RNA synthesis or the nature of RNA transcript (Allfrey 1974).

In conclusion, it can be said that now there is much evidence to support the hypothesis that histones are gene repressors in a non-specific way and acidic nuclear proteins are involved in the positive transcriptional control. However, many aspects of the transcriptional control are still under intensive study, and relatively little is known about the regulatory mechanisms for the transcription of the genetic information.

The hereditary basis of some neuromuscular diseases suggests an involvement of the nucleus in the pathologic process of such disorders and it seems reasonable to suggest that this might be the result of disturbed differentiation. In these diseases the age of onset of clinical symptoms is in the first few months of life, but there is much evidence

(Walton and Gardner-Medwin 1974, Toop and Emery 1974, Vassilopoulos and Emery 1976) to suggest that the pathological process might start in early fetal life.

The aim of the present study was to elucidate possible changes in the main regulators of transcriptional control during normal fetal development of the spinal motor neurones.

In addition, the neurone and nuclear size were estimated since they reflect the state of neurone activity (Edström. 1957, LaVelle and LaVelle 1970). The nuclear size in the developing neurone has been correlated with the functional activity (LaVelle and LaVelle 1970) and in general terms changes in nuclear size may be related to alterations in gene activity (Gurdon 1970, Lewin 1974). As far as the nucleolus is concerned it is now established that it plays an integral role in protein synthesis and particularly in synthesis of RNA species of both the large and small ribosomal subunits. (Smetana and Busch 1974).

The nucleolar size has been suggested as an index of the synthetic activity of a cell (Stowell 1948, Kleinfeld 1957, Busch et al. 1963, Schnedl and Schnedl 1972) and nucleolar changes during development can indicate the rate of RNA synthesis during ontogenesis (Zilles et al. 1976). So, in addition to quantitative histochemistry of the spinal motor neurones during fetal development, the nuclear and nucleolar sizes were examined.

II. Material and Methods

i Material

a. Source of samples - Fetuses used in the present study came from the local hospitals and had been aborted therapeutically (for social reasons). In all fetuses studied there was no family history of muscular or neurological disease. Fetuses were excluded if there was overt evidence or even a possibility of neuromuscular, chromosomal or any other abnormality. All the samples of this study were obtained from fetuses whose abortions had been induced by administration of either intra or extra amniotic physiological saline or Prostaglandin E2 or F2a either alone or together with intravenous Syntocinin. In these cases the death of the fetus preceded abortion by up to 24 hours.

In some specimens the tissue preservation did not appear good on superficial examination, however, subsequent microscopy indicated that the damage had not been so marked as to alter the histological or histochemical profile of the tissue.

b. Determination of the gestational age - This was based on three methods, the menstrual age and the heel-toe/crown-rump length measurements. The gestational age as determined from the first day of the last menstrual period was not always reliable. This sometimes was due to an innacurate date given by the mother, but especially in the case of very young fetuses, because the stage reached in

fetal development is related to the post conceptional rather than to the menstrual age. In the determination of the gestational age more reliance was placed on the heel-toe (Streeter 1921) and the crown-rump (Hamilton and Boyd 1962) length measurements.

c. Samples used - The samples used in this study were sections of the spinal cord. The caudal end (lumbo-sacral region) of the spinal cord was carefully dissected, free from the vertebral column. In fetuses of 10 weeks of gestation or less, the spinal cord alone was too soft to be handled and so the whole lumbo-sacral spinal column was frozen. Since autolysis is rapid in the neural tissue, effort was made to use cords which were as fresh as possible. In all the cases, the second or third lumbar segment (L2-L3) was selected for study by following the associated nerve root.

Finally, sections of the fifth or sixth cervical segment (C5-C6) from four adult spinal cords were also examined.

ii Methods

a. Processing of samples - To minimize autolysis, the spinal cord was removed and frozen within 12 hours of the delivery of the fetus. The samples were attached to chucks by means of Ames OCT and were frozen in iso-pentane chilled in liquid nitrogen. Sections 15 μm in thickness were cut at -20^{o} to -25^{o}C on a "Slee" retracting microtome and attached to glass slides at room

temperature. The slides were allowed to dry for 10-30 minutes at room temperature and in most cases stained the same day, otherwise they were stored overnight at -70°C.

In order to reduce the variation in the intensity of stain due to alterations in the staining procedure, sections of frozen white blood cells were used as controls. The white blood cells were isolated as follows:

Dextran solution (6% Dextran Wt./Vol. in 0.9% saline) and 50% Glucose (Wt./Vol.) prepared daily by adding 0.25 ml. of glucose to 10 ml. Dextran, was added in the proportion of one part to four parts of blood (Vol./Vol.) and mixed with gentle inversion, taking care to avoid frothing. The tube was covered and placed in at an angle of 45° at room temperature for 30 minutes. The supernatant plasma which contained the leucocytes was removed and centrifuged at 300g. for 5 minutes.

After the isolation, a core of white blood cells was embedded in a small block of 7% Agar and then frozen using the same procedure for the specimen preparation. Sections of this block, 15 μm in thickness were mounted on the same slide as the specimen and so subjected to exactly the same procedures.

b. Staining techniques - The following stains were used for the study of the nuclear components of the spinal motor neurones during fetal development.

F E U L G E N-S C H I F F reaction for DNA This method was chosen because it is simple, sufficiently specific for DNA and in addition it would facilitate comparison with other published data from quantitative studies.

Cryostat sections were placed in acetic-ethanol (I:3 V/V) for 10 minutes. After dehydration in alcohol they were rinsed in 1N hydrochloric acid and plunged in 1N hydrochloric acid at 60°C for 6 minutes. (The hydrolysis unmasks the aldehyde groups in the DNA which can then react with the Schiff reagent forming a purple colouration). The slides were then transferred to hydrochloric acid at room temperature and to Schiff's reagent in the dark for 60 minutes. Afterwards they were rinsed in three changes of freshly prepared bisulphate solution and washed in distilled water before dehydration and mounting in DePeX.

F A S T G R E E N reaction for Histones The alkaline Fast Green method (Alfert and Geshwind 1953) was chosen in preference to the other methods available for staining of basic nuclear proteins (Histones) because of its reliability after formalin fixation (Cowden 1966) and its adaptability to quantitative studies.

Frozen sections were fixed for 2-2.5 hours in 10% neutral formalin. The slides then were immersed

for 15 minutes in 5% aqueous trichloro-acetic acid (TCA) at 100°C in order to unmask the basic groups. After washing in three changes of 70% alcohol (10 minutes each) and in distilled water, the slides were stained for 30 minutes, at room temperature, in 0.1% aqueous Fast Green adjusted to pH 8.0 to 8.1 with the minimum amount of NaOH. (At this pH the groups responsible for staining are the guanidine groups of arginine and the ε-amino groups of lysine). The slides were washed in distilled water and transferred directly to 95% alcohol. After dehydration they were mounted in DePeX.

T O L U I D I N E B L U E method for Acidic Nuclear Proteins For the demonstration of the acidic proteins of the nucleus the method of Toluidine-blue at pH 9.0 by Smetana and Busch (1966) was chosen.

The sections were fixed in 10% neutral formalin, prepared according to Lillie (1954) for 2 hours. After the slides were washed in water, nucleic acids were extracted with 5% aqueous trichloro-acetic acid (TCA) in a boiling water bath (at 100°C), and then they were washed in absolute alcohol for 3-5 minutes and dried. The slides were stained with 0.5% Toluidine blue 0 at pH 9.0 (The pH was adjusted with 0.1 N NaOH). After a short wash in running water the slides were

dried and mounted in DePeX.

Combined P H E N A N T H R E N Q U I N O N E and F A S T G R E E N method for Lysine-rich Histones Since there does not appear to be any reliable, specific method for the lysine-rich histones, a combination of two stains was developed to demonstrate this nuclear component. The slides were stained first with phenanthrenquinone (PQ) solution in order to block the guanidine groups of arginine as described by Magun and Kelly (1969) and subsequently slides were stained with 0.1% aqueous Fast Green (as described above) for demonstration of the remaining histones. (The ε-amino-groups of lysine).

The sections were fixed in 10% neutral formalin for 2-2.5 hours. The nucleic acids were removed with 5% trichloro-acetic acid (TCA) at 100°C for 15 minutes and then they were washed in 3 changes of 70% alcohol followed by distilled water. After rinsing in three changes of absolute alcohol (1 minute each), they were put in PQ solution for 10 minutes. (The solution was made up by 1 part of 0.5%N NaOH, 4 parts of absolute alcohol and 1 part of freshly prepared solution of 1% PQ in dimethyl-formamide). The slides were rinsed in three changes of 95% alcohol (1 minute each) and then were stained in 0.1%

aqueous Fast Green at pH 8.0-8.1. After staining the slides were dehydrated and mounted in DePeX.

c. Quantitative Histochemistry Neurones examined were chosen for study at random providing that their identification was clear, there was no overlapping of nuclear fragments and the nucleus was intact, on the basis of their size and location. The neurones chosen were those with large somata (α-motor neurones) and which were located in the lateral aspect of the lumbar ventral column of both halves of the spinal cord. (Lamina IX according to Gray 1973). The optical density of at least 50 neurones was measured (The background optical density of the cytoplasm was deducted automatically). In addition, the density of at least 30 polymorphonuclear nuclei (lying on the same slide) was measured by the same technique. The result for each neurone was expressed as optical density per unit area. Mean values from 50 nuclei were calculated and were expressed as the proportion of the optical density per unit area of leucocytes. The values for each feature studied were grouped according to gestational age into seven groups each of three weeks.

A quantitative estimation of the histochemical stains was obtained by use of a scanning microdensitometer (Vickers M86). Microdensitometry is the measurement of light absorbance by objects observed through a microscope.

Since with compounds that obey Beer's law the absorbance is proportional to the amount of the compound, the light absorbance can be used to measure the amount of various substances in cells or parts of cells. It is also possible to measure the area of an object simultaneously by counting the number of "points" which absorb light. In the present study the wavelength per maximum absorption of each stain was estimated in an ultra-violet spectophotometer (Unicam SP 800). For the Feulgen stain the maximum absorption was at 560nm, for Toluidine blue at 590nm and for Fast Green at 625nm.

The scanned integrated density measurement with background setting was chosen, because this is the most suitable method for extensive objects and for measurements where there is insufficient clear background area for a background subtractive scan.

Since the whole field of quantitative cytology is dependent on the elimination of various errors, care was taken to minimise every possible source of error.

Stochastic errors These errors displace the measurement in a generally random manner. Typical stochastic errors in microdensitometry are due to random variations in photomultiplier sensitivity or scanning lamp output and also to variations in scanning speed and in electronic, optical or specimen noise. In the present study, repeated measurements of the same neurone nucleus were performed

and the results are as follows:

	No. of measurements	Mean ± S. D.	Standard error	Coefficient of variation
DNA	20	0.62±0.009	0.002	1.45
Total histones	20	1.09±0.035	0.008	3.21
Acidic nuclear proteins	20	1.18±0.049	0.011	4.15
Lysine-rich histones	20	0.91±0.040	0.009	4.39

Systematic errors These errors displace the mean of measurement in a positive or negative manner so that unless taken into account they have a definite effect on the accuracy of measurements (1) Distribution error The effect of this error is adequately eliminated by the scanning microdensitometric methods themselves. This is mainly due to the fact that the distribution of a substance in a cell is not homogeneous. The scanning microdensitometer measures the absorbance of very small areas, not themselves subject to marked distribution error, which are electronically summed to give a figure representative of the total amount of the material in the specimen. (2) Glare This arises from unwanted reflections at air-glass surfaces in the optical system or by dispersion of light at unclean surfaces. There is a close relationship between the ratio of the size of the illuminated measuring field to the specimen size and the percentage of glare included in the densitometric

measurements. A reduction of glare was attempted by closing the field iris to a size just larger than the measuring field. In any case, the expected glare in a Vickers M86 microdensitometer lies between 1% and 3%. (3) Spot size and focus error Since an increase in the size of the measured area (spot) will result in a reduction in the integrated optical density, the smallest possible spot was used. Defocusing of the specimen has the same effect, so care was taken in specimen focusing particularly when high power objectives were used.

In summary, cytophotometry in the present study was performed using a Vickers M86 scanning microdensitometer with a x100 apochromatic objective. The apochromatic microscope condenser was used dry at full aperture. The field illuminated was limited by a field stop to reduce glare and the background density was always set to zero.

d. Neurohistometry - Toluidine blue-O (pH 9.0) stained sections were used. The motor neurones were photographed and then examined at a final magnification of x1000.

The size of the motor neurone soma was estimated from the major (X) and minor (Y) axes of the neurone passing through the nucleolus. In measuring the axes, care was taken not to include dendrites; however, some allowances were made for the bases of dendrites (Fig. 1.1).

Figure 1.1. Adult motor neurone depicting the method of measurement of major and minor axes of the neurone (X and Y) and its nucleus (X' and Y'). Frozen section stained with Fast Green (x 250).

A number of equations have been proposed for the calculation of the neurone body volume. The one proposed by Schadé (1964) as giving the best approximation for the motor neurone volume was chosen. The equation is:

$$\text{Neurone soma volume} \quad V_N = 1.04 \times \frac{1}{6} \pi XY \cdot \sqrt{XY}$$

The volume of at least 50 neurones per fetus was estimated and the mean neurone size for each fetus was computed.

Measurements of the neurone nuclear size were made by estimation of the long (X') and short (Y') "diameters" of the nucleus. These two "diameters" were multiplied and the square root extracted to give a computed diameter $d = \sqrt{X'Y'}$. The nuclear volume was estimated (on the assumption that the nucleus is spherical) using the formula:

$$\text{Neurone nuclear volume} \quad V_n = \frac{1}{6} \pi d^3$$

Finally, for the estimation of the nucleolar size in an attempt to minimise the possible error from measuring its small diameter, nucleoli were traced and areas were measured by planimetry. Nucleolar volumes were calculated on the assumption that nucleolus is spherical.

In addition, since the nucleus is part of the developing neurone it was considered that the "allometric equation" (Huxley 1932, Bertalanffy 1960) might provide a better

representation of their relative growth. (see Chapter 6).

III. Results

Quantitative histochemistry As it can be seen from Table 1.I and Fig. 1.2, the optical density per area unit of DNA remains constant through the gestational periods examined.

The total histones show a significant increase in the 12-14 weeks period and after 15-17 weeks their density remains constant. (Analysis of variance showed that this increase was statistically significant. $F=2.46$ and $p < 0.05$). This increase in the amount of total histones is largely due to an increase ($F=2.03$ and $0.05 < p < 0.1$), the lysine-rich fraction remaining unchanged during the periods examined. (Fig. 1.3).

The acidic nuclear proteins seem to exhibit the same pattern during development as the total histones. At 12-14 weeks there is a considerable increase in the density per unit area which later falls to its original level. ($F=2.62$ and $p < 0.05$).

In conclusion, it seems that at 12-14 weeks of gestation the acidic nuclear proteins and the arginine-rich histones increase significantly in comparison to the values observed

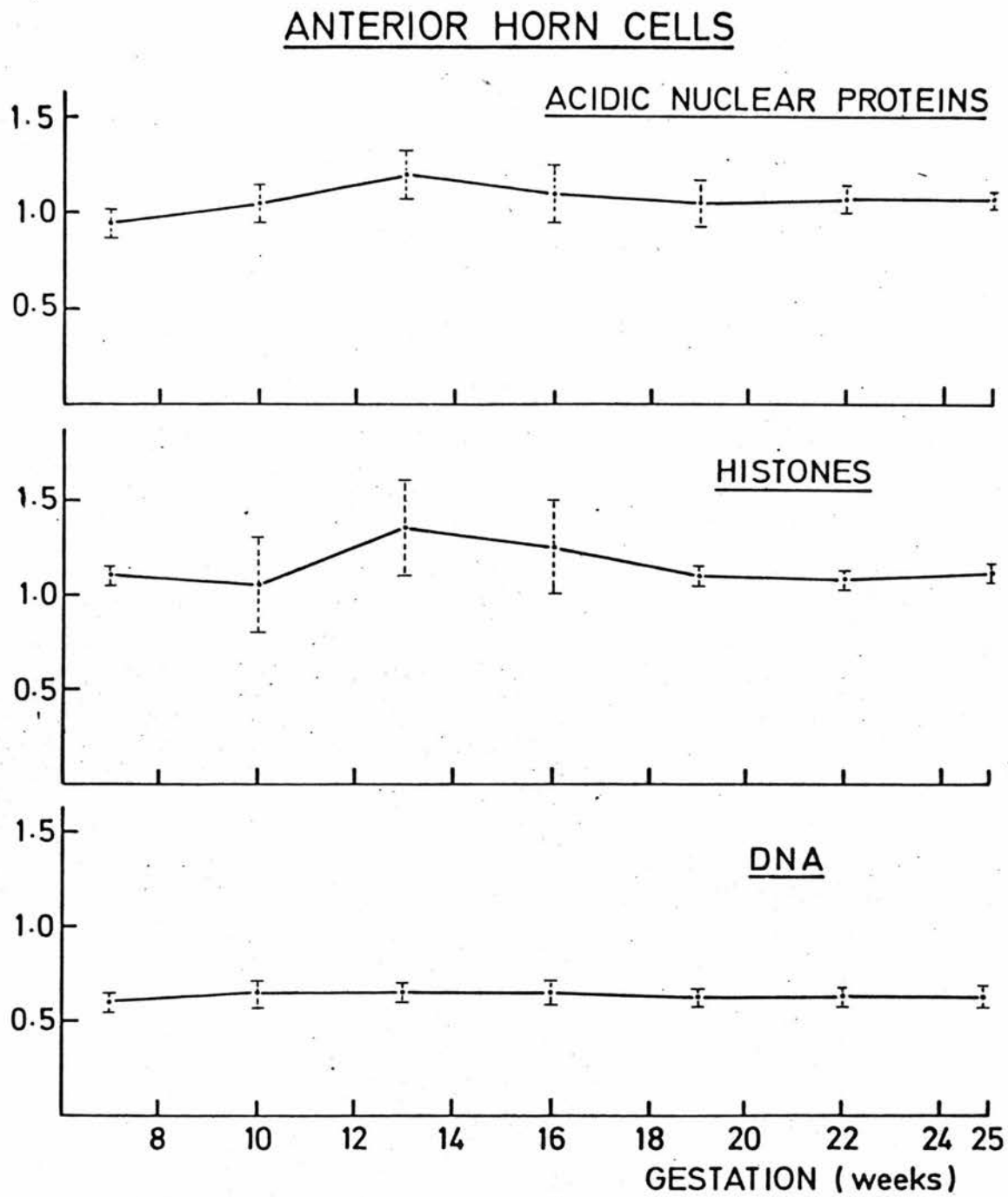


Fig. 1.2 Mean density per unit area of DNA, histones and acidic nuclear proteins in fetal anterior horn cell nuclei for different periods of gestation. (The dotted vertical lines represent a standard deviation on either side of the mean value)

Gestation (weeks)	No. of fetuses	D.N.A.	Histones			Acidic Nuclear Proteins
			Arginine-rich	Lysine-rich	Total	
6-8	4	0.61±0.05	0.20±0.05	0.89±0.02	1.10±0.04	0.95±0.07
9-11	5	0.66±0.06	0.15±0.10	0.91±0.14	1.06±0.25	1.04±0.11
12-14	10	0.64±0.08	0.35±0.25	0.98±0.09	1.35±0.25	1.20±0.13
15-17	8	0.64±0.07	0.32±0.21	0.93±0.09	1.25±0.24	1.09±0.14
18-20	6	0.62±0.05	0.14±0.05	0.95±0.08	1.10±0.04	1.06±0.13
21-23	5	0.63±0.04	0.17±0.06	0.91±0.09	1.08±0.05	1.07±0.08
24-26	4	0.65±0.05	0.14±0.06	0.94±0.05	1.09±0.02	1.07±0.02

Table 1.I. Mean density per unit area of DNA, histones and acidic nuclear proteins in fetal anterior horn cell nuclei for different gestational age groups. (Results are expressed as mean ± standard deviation).

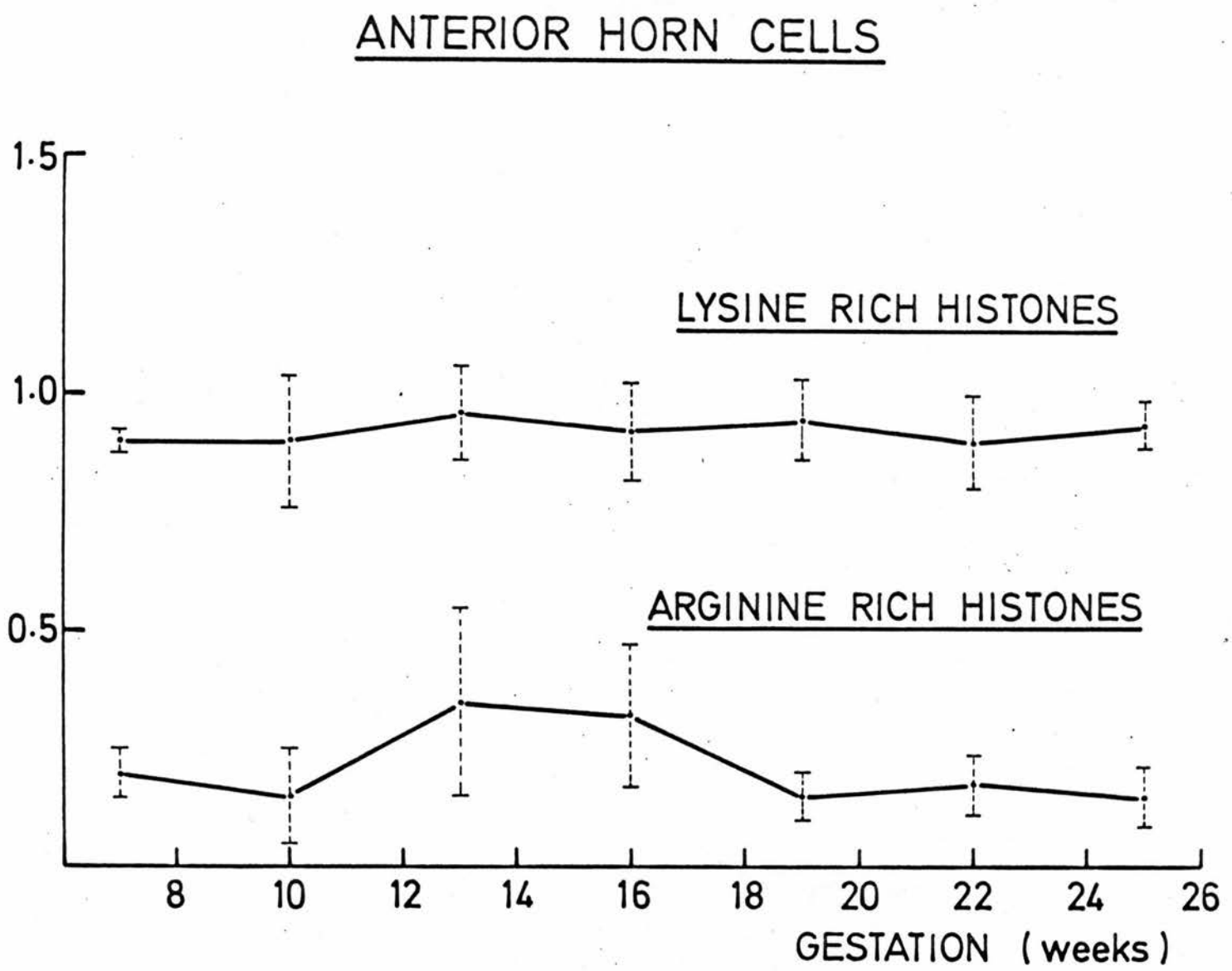


Fig. 1.3 Mean density per unit area of lysine-rich and arginine-rich histones in fetal anterior horn cell nuclei for different periods of gestation. (The vertical lines represent a standard deviation)

before and after this period.

Neurohistometry

The results of this part of the study are given in Table 1.II. The neurone size as well as the nuclear size (Fig. 1.4 and 1.5) develop in a linear manner during the fetal development, with exception the period of 12-14 weeks of gestation. At this stage a discontinuity is observed. Statistical analysis showed that this discontinuity is due to an increased neurone and nuclear size at 12-14 weeks rather than to a decrease at 15-17 weeks period. The statistical test used to detect if this increase differed significantly from linearity of the other values showed that the increase observed at 12-14 weeks period was just below the level of statistical significance.

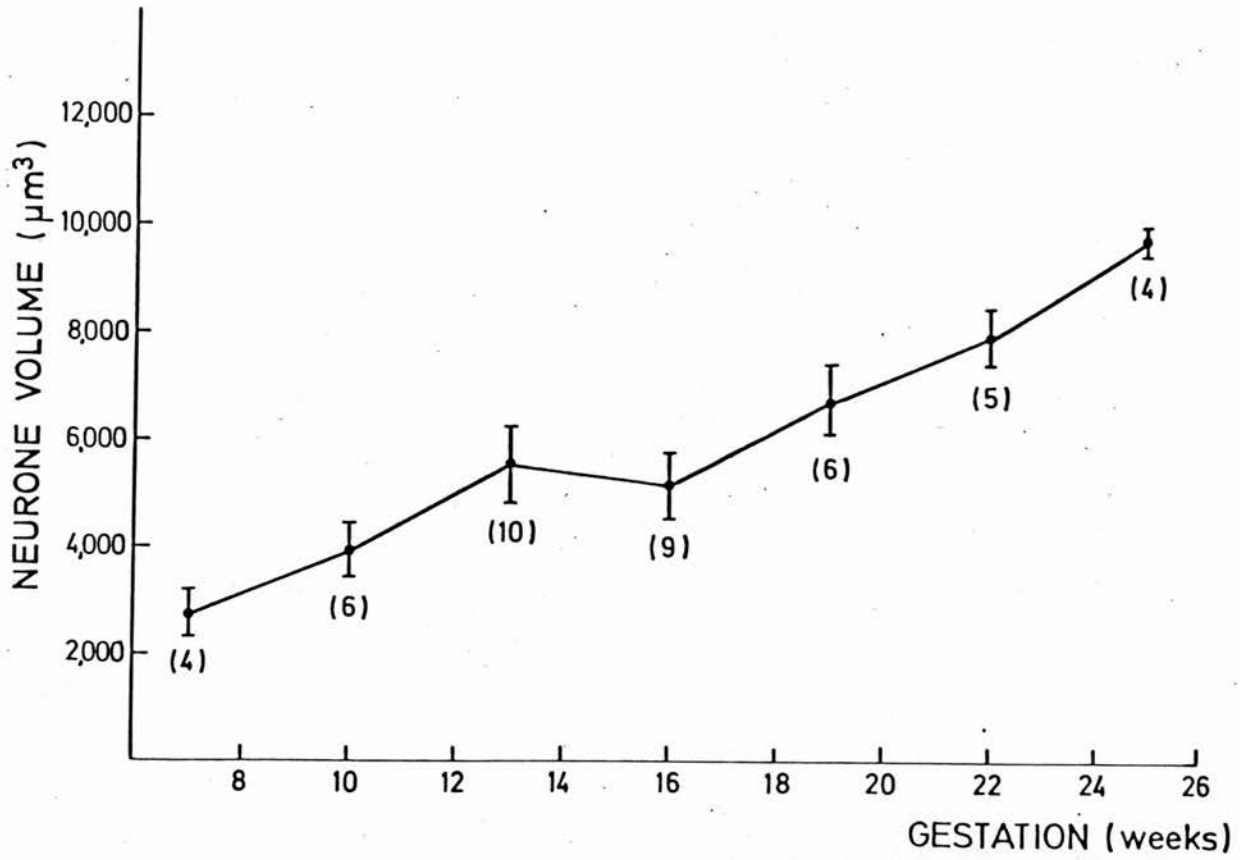
Apart from the 12-14 week period, the other values on neurone and nuclear volume are correlated linearly with the gestational age. The equations describing this correlation are:

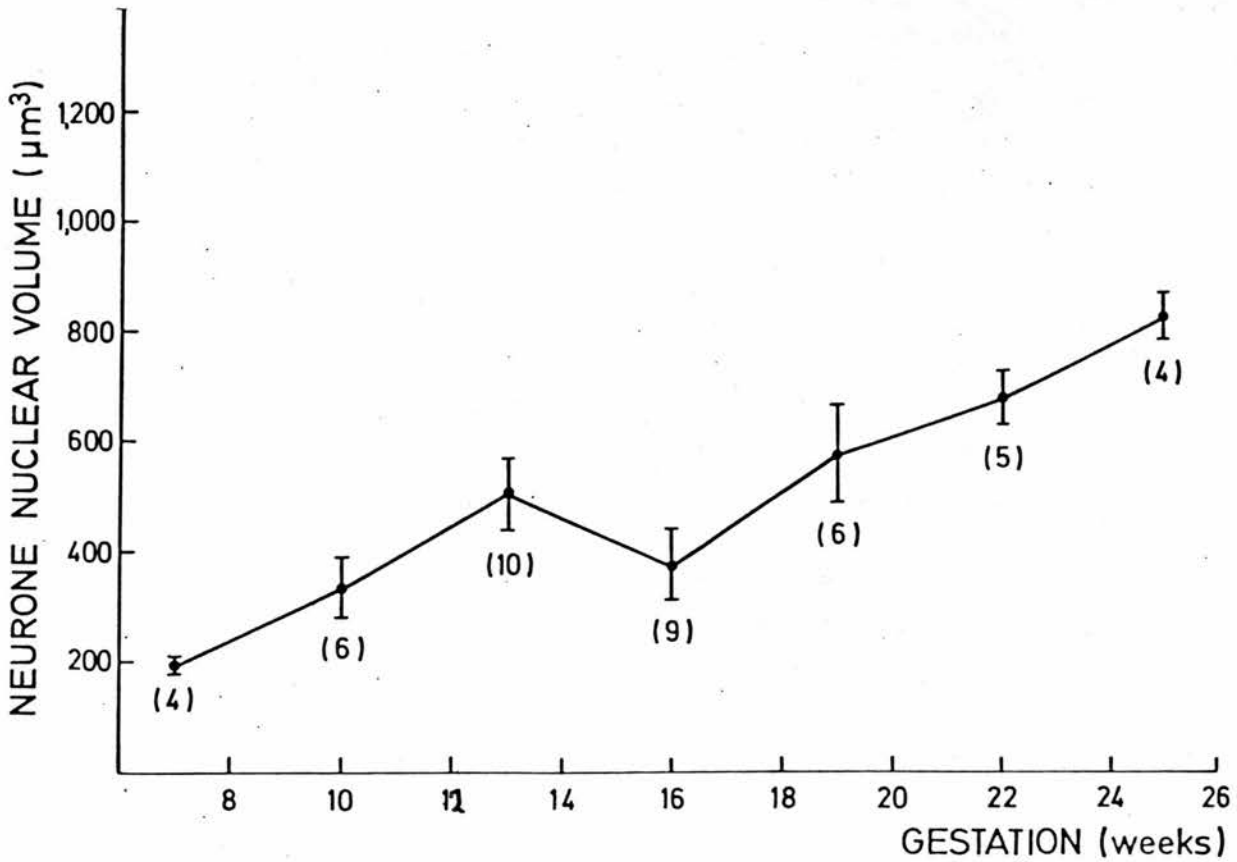
Motor neurone soma $Y = 369.0\ (X) - 45.6\ (r = 0.97)$

Motor neurone nucleus $Y = 33.2\ (X) - 50.9\ (r = 0.98)$

The allometric equation for the relative growth of the nucleus and cytoplasm is

$$Y = 0.0252 \cdot X^{1.136}$$





Figs. 1.4 (top) and 1.5 (bottom) Fetal anterior horn cell and its nuclear volume during gestation. (The vertical lines represent an S.D. on either side of the mean and the figures in brackets the no. of fetuses in each group).

Gestation (weeks)	Neurone size (μm^3)	Nuclear size (μm^3)	Nucleolar size (μm^3)
6-8	2744 ± 859	193.2 ± 16.1	7.7 ± 3.8
9-11	3956 ± 1123	334.8 ± 136.1	10.1 ± 2.1
12-14	5545 ± 2287	504.1 ± 204.9	11.7 ± 2.1
15-17	5133 ± 1791	376.6 ± 193.6	23.1 ± 3.7
18-20	6732 ± 1702	577.8 ± 219.2	26.7 ± 3.5
21-23	7931 ± 1114	678.2 ± 105.9	20.6 ± 3.7
24-26	9768 ± 366	830.0 ± 83.2	—

Table 1.II. Neurone, nuclear and nucleolar volumes of fetal anterior horn cells for different periods of gestation. (Results are expressed as mean ± standard deviation).

(Y represents the growth of the nucleus and X the growth of the neurone body).

From this equation it can be seen that during fetal development the nuclei of the spinal motor neurones develop faster than the neurone soma.

The nucleolar volume increases linearly until 12-14 week period when a dramatic increase is observed. (Fig. 1.6). Thereafter there is apparently no further increase in nucleolar size.

In conclusion the changes in acidic nuclear proteins and the arginine-rich histones during 12-14 weeks of gestation are paralleled by an increase in the neurone somal, nuclear and nucleolar size. (Plate 1.)

IV. Discussion

Cytodifferentiation is the process by which different cell types in a developing multicellular organism acquire their special features. This seems to be a selective transcription of the DNA template resulting from a complex interaction of nuclear and other components.

It is now well established that the majority of somatic cells are diploid and have an appropriate DNA content. Polyploid cells have been observed in animal tissues, but little is known of this in the human nervous system. There

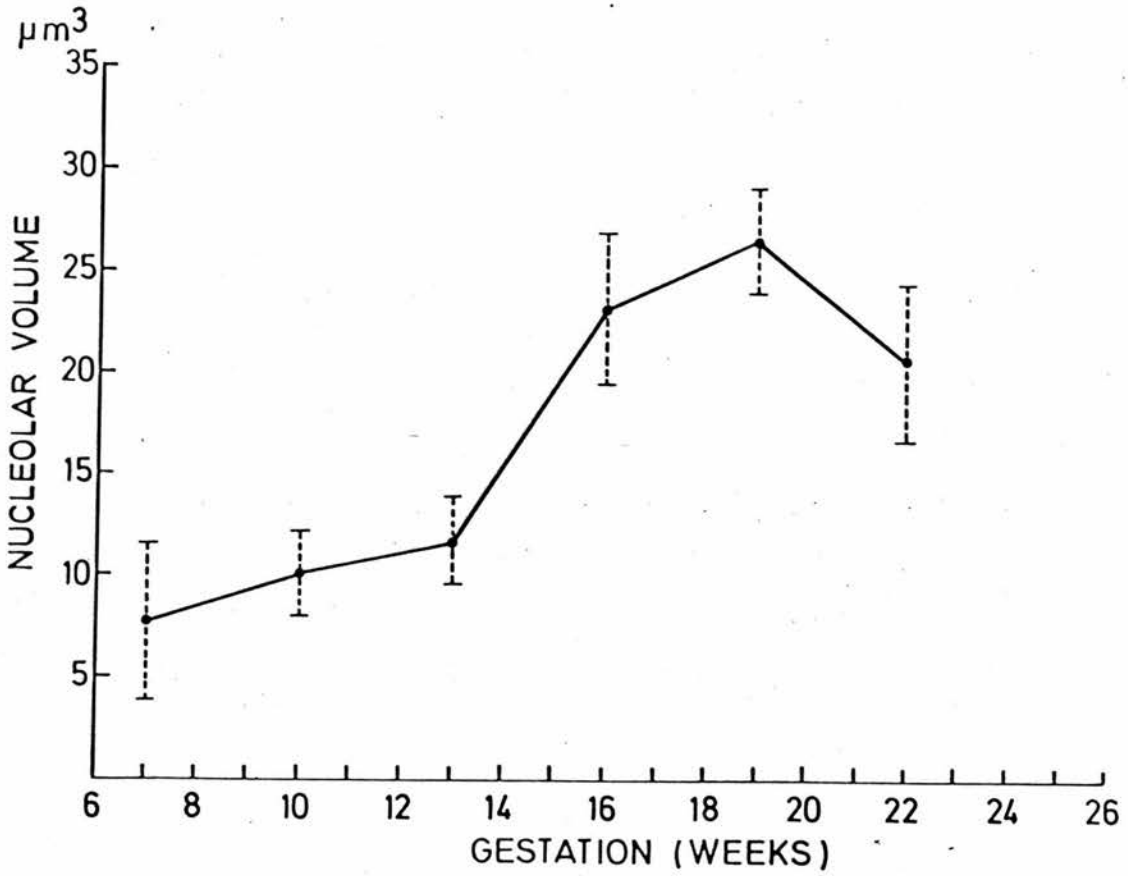


Fig. 1.6 Fetal anterior horn cell nucleolar volume during gestation. (The dotted vertical lines represent the standard deviation of the mean value).

The results obtained from the motor neurone study of four normal adult spinal cords are as follows (overall means $\pm$ standard deviation):

DNA	0.68 ± 0.04
Histones	1.11 ± 0.03
Arginine-rich	0.20 ± 0.05
Lysine-rich	0.91 ± 0.03
Acidic nuclear proteins	1.09 ± 0.03
Neurone size	19630 ± 1433 (μm^3)
Nuclear size	1680 ± 155 (μm^3)
Nucleolar size	69 ± 5.9 (μm^3)

is much evidence to suggest that the nerve cells are diploid although many authors have interpreted their observations as suggestive of polyploidy, Lapham (1965, 1968) found that during the perinatal period the Purkinje cells of the cerebellum and their nucleus enlarge considerably. These changes were correlated with a doubling of the DNA content to a tetraploid state and the author suggested that this DNA doubling was necessary for supplying the increased protein required by the neurone because of the rapid increase of the dendritic tree. A polyploidy has also been reported in the neurones of the dentate nucleus of the human infant (Muller 1962). However, Mann and Yates (1973 a) by a more refined and detailed methodological approach showed that the neurones of the human cnetral nervous system have a diploid DNA content as do the other somatic cells of the human body. The authors suggested that these polyploidies may have resulted from cytophotometric errors and particularly from changes in the non-specific light loss. However, Mann and Yates (1973 b) found classes of polyploid glia within the Purkinje cell layer of the cerebellum and since there is a strict numerical proportionality between the glial types and the Purkinje cell, they suggested that these cells are possibly involved in the process of cerebellar memory. Recently, Bedi and Goldstein (1974) provided further evidence that there are no differences in the amount of DNA present in different leucocyte types and differences previously reported are due to glare and residual distribution error. The present study is concerned with the DNA absorbance per unit area rather than with the absolute DNA content.

The present results suggest that there are no apparent

changes in the amount of DNA of the neuronal nucleus studied during fetal development and this supports Mann and Yates observations.

The observed increase in the total histones, at 12-14 weeks of gestation, is attributable to an increase in the arginine-rich histones and might reflect alterations in the transcriptional process during this period. Several authors have investigated changes in histones during development since these substances may act as genetic repressors.

It has been reported that the development of animal embryos can be arrested by histones (Brachet 1964, Kimmel 1965) and Sherbet (1966) reported developmental arrest and malformations in chick embryos exposed to histones. The mechanism of this action of histones in embryonic development appears to be non-specific (Blaszek and Gyergyay 1965, Kischer and Hnilica 1967).

Biochemically, Dingman and Sporn (1964) found no changes in the DNA:histone ratio and Kischer and Hnilica (1967) reported no changes in the total aminoacid composition of the histones during development. Similar studies in the developing tissues of chick and other animals failed to demonstrate marked changes in the histone composition during embryogenesis (Lindsay 1964, Niedle and Waelsch 1964, Kischer et al. 1966).

Effort has been made to elucidate possible changes of the histone fractions during ontogenesis. Agrell and

Christensson (1965) studying the various histone fractions (arginine and lysine-rich histones) found no changes in the concentrations of these subgroups during chick brain development. However, changes in the distribution of histones in dorsal root ganglion neurones during development has been reported (Kornguth and Tomasi 1968) and a possible role of lysine-rich histones in the transcriptional control has been noted (Kinkade 1969, 1971). On the other hand, the role of arginine-rich histones in the process of differentiation has been confirmed by Bloch and Hew (1960) and Gledhill et al. (1966) who showed that during spermatogenesis there is a shift in the histone population from lysine-rich to arginine-rich histones. Certain changes in the concentration of arginine-rich histones have also been found during the process of ageing (Pyhtilä and Sherman, 1968).

The results indicated an increase in the amount of the acidic nuclear proteins at 12-14 weeks of gestation. As previously mentioned, there is little doubt about the positive role of the acidic nuclear proteins in the transcriptional control. These results provide additional evidence for an alteration in the control of transcription during the 12-14 week period.

The neurohistometric data provided further information on the developmental pattern of the motor neurones. The neurone and the nuclear size also increased considerably

at the 12th to 14th week of gestation. Changes in cell and nuclear size have been observed in a number of conditions (see Chapter 5) and they might be regarded as alterations of the nucleo-cytoplasmic relationships. Much information about changes in this relationship has been gained from the nuclear transplantation (Gurdon 1968, 1970) and cell fusion (Harris 1970) experiments. Nuclear enlargement in particular, seems to be due to a marked influx of pre-formed proteins and this enlargement is often accompanied by a general change in nuclear activity (Lewin 1974, Goldstein 1974).

Nucleolar enlargement has also been observed under various conditions such as liver cell in partial hepatectomy and after administration of carcinogenic drugs (Stowell 1958, Buscher 1963), in megaloblasts of pernicious anaemia (Ayres 1948, Busch et al. 1963) and in neoplastic cells of experimental animals (Ferreira 1941). This enlargement is believed to be a sign of increased synthetic activity of the cell.

In motor neurones of experimental animals Edström (1957) found that the nucleolar size increased after prolonged activity and this has been explained as a reflection of increased protein synthesis in response to increased functional demands. The nucleolar enlargement observed in this study is possibly suggestive of an associated increase in the functional activity of the developing motor neurone at 12-14 weeks of gestation.

In conclusion, the results of both the histochemical and histometric studies indicate that at the 12th to 14th week of gestation a change in the nuclear composition and activity may occur.

In the human fetus, spontaneous motor activity begins at 9-10 weeks of gestation, but at approximately the 14th week movements begin to be far less stereotyped and more individualistic (Hamilton and Boyd, 1962). It has been suggested that there is a correlation between fetal movements and the appearance of acetyl-cholinesterase (AChE) activity in a number of animals. Duckett and Pearce (1969) examining the developing neurones of human fetuses reported that at 12-14 weeks of gestation motor neurones are identifiable by Haematoxylin and Eosin and Nissl staining techniques and this coincides with the appearance of reflex movements of the fetal lower limb. Toop and Jackson (1973) reported a similar increase in AChE activity at 11-12 weeks and they noted that at this stage light and dark types of motor neurones can readily be identified and are grouped in columns as in the adult spinal cord. This confirms Romanes' (1940-41) observation that cell columns have attained an adult arrangement as early as the 14th week of fetal life. Toop and Jackson (1973) did not regard their results as supporting the suggestion that the development of the motor neurones is related to the muscle fibres development, since in the latter, histochemical changes take

place between 11 and 30 weeks of gestation.

The differentiated motor neurone plays an important role in the myelination process by supporting and interacting with the lemma cells. So, further support for the suggestion that the 12-14 week period is important for the differentiation of the motor neurone, comes from the observation that myelination of the motor nerve roots first appears at 15-16 weeks of gestation (Elliott 1969).

Finally, Wolf et al. (1975) recently confirmed earlier observations by reporting that fetal motor neurones stain more intensely for AChE at 12-14 weeks of gestation and a similar increase in succinic dehydrogenase (SDH) activity is observed at the same period.

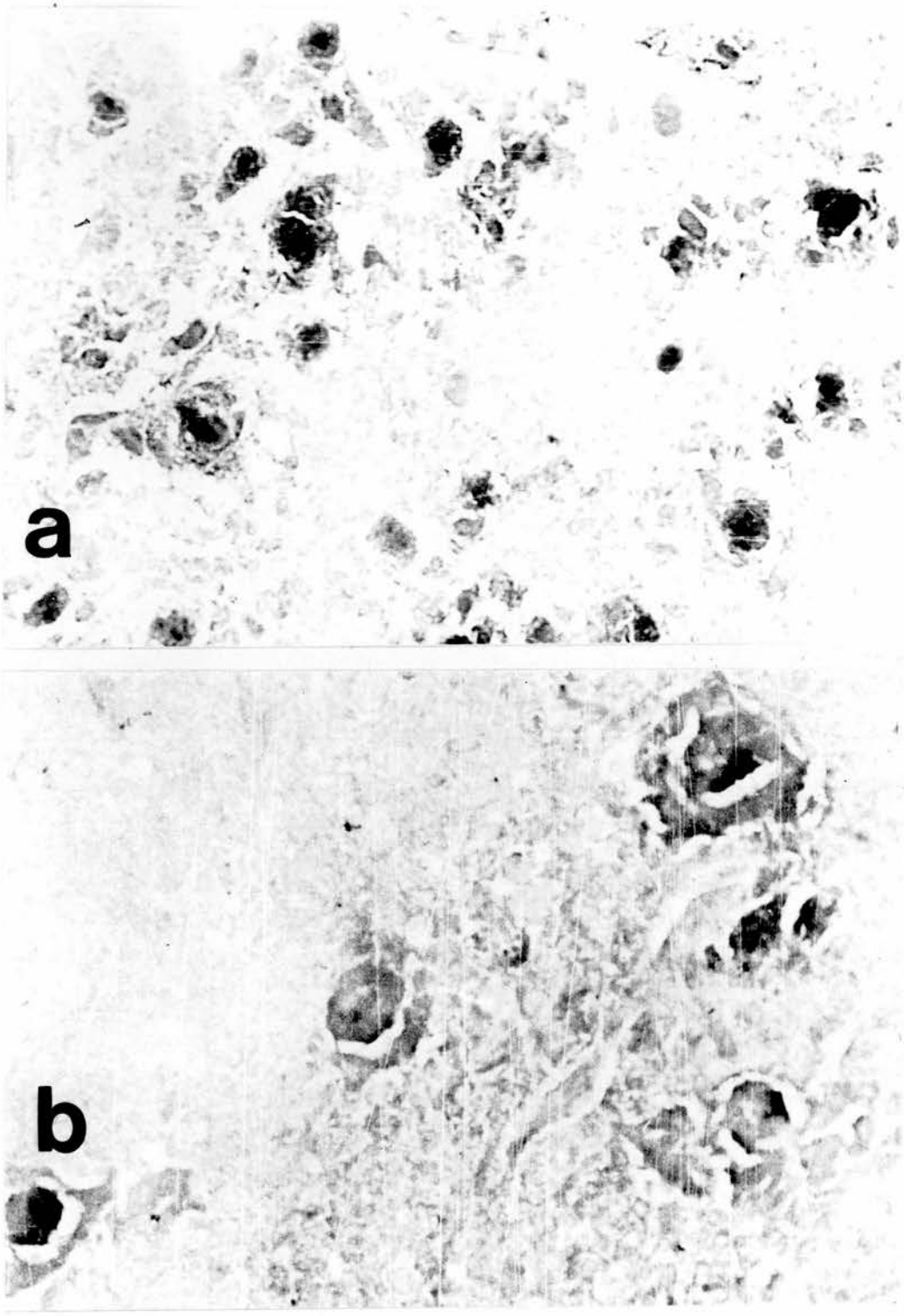


Plate 1. Anterior horn cells in transversely sectioned spinal cord from a fetus of 12 weeks of gestation (a) and 24 weeks of gestation (b). Cryostat sections stained with Fast Green (x 700).

CHAPTER 2

Muscle nuclear changes

during development

I. Introduction

II. Material and Methods

- i Material
  - a. Source of samples
  - b. Determination of the gestational age
  - c. Samples used
- ii Methods
  - a. Processing of samples
  - b. Staining techniques
  - c. Quantitative histochemistry
  - d. Muscle histometry

III. Results

Quantitative histochemistry
Muscle histometry

IV. Discussion

C H A P T E R T W O

I. Introduction

In modern biology cell development is seen as a sequence of qualitative and quantitative changes in the synthesis of macromolecules. Recently, exceptional advances have been made in the understanding of the role of the contractile molecules in muscle cell structure and function (Huxley 1969, Lowey et al. 1969). The structure is believed to be closely linked to function of the muscle and its two unique characteristics (i.e. The functional association with the nervous system and the presence of well characterised proteins related to contraction).

The general scheme of muscle development is now well understood. (Konigsberg 1965, Holtzer 1970, Herrmann et al. 1970, Fischman 1970). The multinucleated muscle fibre is formed by the cytoplasmic fusion of the non-dividing, spindle-shaped population, the promyoblasts or presumptive myoblasts. There is now much evidence (Okazaki and Holtzer 1965, Yaffe 1969, Bischoff and Holtzer 1969) that myoblasts undergoing fusion have ceased to synthesise DNA and can be considered as a postmitotic population. Synthesis of the contractile proteins seems to begin after fusion and the inverse relationship between the synthesis of myofibrillar protein and DNA replication has received great emphasis. As Holtzer (1970) stated "no cell going through S, G_2 or M is making, or has made, these contractile proteins" and "only cells in some phase of G_1 may initiate the synthesis of contractile proteins".

A further understanding of myogenesis has been aided by a comparison of similar and divergent developmental mechanisms of muscle development in distinctive muscle types as well as genetically determined and experimentally produced abnormal forms of muscle development.

Of the human cell types, skeletal muscle is exceptional (along with the syncytiotrophoblast of the placenta) in its syncytial character. It is generally accepted that this syncytium is formed by fusion rather than "amitosis". The

strongest support for this comes from studies of DNA synthesis. Quantitative estimations of the DNA content of animal muscles using Feulgen's method showed that all nuclei within myotubes have DNA values consistent with diploidy. (Lash et al. 1957, Firket 1958, Basleer 1962, Cox and Simpson 1970). These studies demonstrated the absence of DNA doubling within myotube nuclei. Evidence for cell fusion has also accumulated from morphological, autoradiographic and cytogenetic studies. (Fischman 1972).

The most interesting aspect of muscle fibre differentiation is the rapid accumulation of contractile proteins in the multinucleated fibre. This accumulation is detectable only after cell division has ceased (Stockdale and Holtzer 1961) and the presence of these proteins at a particular phase in the development process may reflect initiation of synthesis of particular proteins, an increased rate of synthesis, or stabilization of the protein (Konigsberg 1965). It has been reported (Ross and Jans 1968) that the concentration of RNA in the cytoplasm of one of the cells in each fusing pair temporarily increases. This suggests that possibly this extra cytoplasmic RNA is mainly ribosomal and it is involved in initiating the metabolic process which cause and complete cell fusion.

In recent years, much emphasis has been placed upon developing techniques for the detection of characteristic gene

products in differentiating cells (Holtzer et al. 1957, Coleman and Coleman 1958). With such methods, attempts have been made to correlate the synthesis of the contractile proteins of the muscle fibre with embryonic age, cellular proliferation, cytological structure, and myotube formation, but the results depend greatly on the sensitivity of the method and its degree of precision. At the present time I do not think it is possible to localise with sufficient accuracy the moment of the appearance of contractile proteins in the differentiating muscle.

Another difficulty in studying muscle cell differentiation is the problem of which of the contractile proteins are differentiative determinants. For instance if, hypothetically, actin and myosin are synthesized at appreciably different time, it is very difficult to decide at which stage the cell was differentiated.

Finally, the problem of muscle cell differentiation has been further complicated by the recent report of Ishikawa et al. (1969) that actin like filaments are present in non-muscle cells within chick embryos. Thus, our assumption that the synthesis of contractile proteins denotes muscle differentiation may have to be re-evaluated in the light of the fact that other cells, not usually considered contractile, may synthesize protein similar to those previously considered to be specific to muscle.

The development of multinucleated fibres resulting from

fusion of myoblasts is accompanied in vivo by a number of phenomena such as cessation of DNA synthesis, reduction of RNA synthesis (Marchok 1966), changes in the RNA molecules synthesized (Herrmann et al. 1970) and the appearance of contractile proteins (Fischman 1970). However, many aspects of muscle cell differentiation remain unclear. The role of the nucleus in the control of muscle differentiation is not well known. At the moment of myoblast fusion and later, during the differentiation of the muscle cell, morphological alterations of the shape of nuclei with a general chromatin condensation have been observed. (Marchok and Wolff 1968, Fischman 1970). These modifications seem to be due to some physicochemical changes in DNA structure, which can probably account for the DNA replication arrest as well as for the decrease of RNA synthesis. Changes in the ionic concentration inside the cell (Franke and Schinko 1969) have been considered as responsible for these modifications. What the role of the nuclear proteins is in these modifications and generally in muscle cell differentiation remains to be determined.

The first part of the present study was undertaken in order to elucidate possible changes in the regulators of the gene expression (histones and acidic nuclear proteins) during normal fetal development.

During development, tissue growth is due to an increase in both the size and the number of cells. Cells may increase

in size only up to a genetically determined limit, characteristic for the tissue and this increase is accompanied by an increase in nuclear size in order to maintain an almost constant nucleo-cytoplasmic ratio. The relative constancy of this ratio in all tissues, indicates the fundamental importance of the nucleo-cytoplasmic interactions for the maintenance of normal cell life.

Striated muscle along with the syncytiotrophoblast of the placenta are regarded as the only two syncytial tissues of the mammalian body (Muir 1970). The nucleo-cytoplasmic ratio during normal muscle growth does not appear to have been documented. Some evidence on the pattern of muscle growth has come from the animal studies of Enesco and Puddy (1964), who reported an increase in the number of nuclei in the post-natal growth of rat muscle. However, muscle development in the rat may differ from that of humans since rat postnatal growth tends to be due to an increase in cell size rather than an increase in cell number (Enesco and Leblond 1962, Cheek 1968). The two possible mechanisms for the maintenance of the nucleo-cytoplasmic ratio during human muscle development are an increase in the size and/or number of nuclei.

Detection of changes in this ratio would possible prove helpful in understanding not only normal muscle growth, but also the muscle response to disease processes. Therefore, an estimation of the muscle nuclear size during fetal and post-natal muscle development as well as an estimation of the

number of nuclei per muscle fibre in postnatal development was attempted. The preliminary results have already been published. (Vassilopoulos et al. 1976c).

II. Material and Methods

i Material

a. Source of samples The material was obtained from normal fetuses aborted for social reasons, by administration of prostaglandins. Fetuses with an evidence of abnormality or with a family history of neuro-muscular disorder were excluded (see Chapter 1). Effort was made to obtain the specimen as soon as possible after abortion and the specimen was used for this study only after careful microscopic confirmation that the histological and histochemical profile was unaffected by autolytic change.

b. Determination of the gestational age This was based on three methods, the menstrual age, the heel-toe and the crown-rump length measurements (see Chapter 1).

c. Samples used The gastrocnemius muscle from 44 fetuses was dissected and frozen within 12 hours. Twenty four normal human muscle biopsies (quadriceps femoris) were also examined. These biopsies were divided into four groups according to the age of the individual. In the first group the age range was 1-2.5 (mean 1.8) years, in the second

12-30 (mean 21.8) years, in the third 32-55 (mean 45.7) years and in the fourth 60-71 (mean 65.1) years.

ii Methods

a. Processing of samples The gastrocnemius muscle was frozen as soon as possible after receipt. The samples were attached to chucks and plunged in isopentane chilled in liquid nitrogen. Sections 10μm in thickness were cut at -20^{o} to -25^{o}C on a "Slee" retracting microtome. As with the spinal cord, the sections were attached to glass slides, dried and either stained or stored overnight at -70^{o}C. Sections 10 μm thick of white blood cells embedded in Agar were also used on the same slides as controls in order to minimize alterations due to the staining procedures. The isolation of the white blood cells and the whole procedure was described in Chapter 1, page 22.

b. Staining techniques For the staining of the nucleus of the developing muscle fibre, four stains were used. The Feulgen-Schiff reaction was used for the DNA, the Fast Green reaction for the total histones, the Toluidine-blue method for the Acidic Nuclear Proteins and finally a combination of Phenanthrenquinone and Fast Green reaction for the demonstration of the lysine-rich histones. The amount of the arginine-rich histones was calculated by subtraction of the lysine fraction from the amount of total histones. Details of the staining procedures were given in Chapter 1. (pages 22-26)

c. Quantitative Histochemistry A quantitative

assessment of the nuclear components during development was performed, by use of a scanning microdensitometer (Vickers M86). The wavelength chosen for maximum absorption were the same as those used previously. (Feulgen = 560 nm, Toluidine blue = 590 nm and Fast Green = 625 nm). Only sarcolemmal nuclei were chosen for measurement. Care was taken to exclude fibroblast, pericyte and endothelial cell nuclei. The optical density of the nucleus of at least 50 muscle fibres was measured with a background subtractive scan. In addition, the optical density of at least 30 polymorphonucleocyte nuclei lying on the same slide was measured. In both cases (muscle fibre nuclei and polymorphonucleocyte nuclei) the results were expressed as optical density per unit area. The mean value from the muscle fibre nuclei was corrected by considering the mean value of leucocytes as being equal to 1.

Care was taken to minimize the systematic error, i.e. the distribution error, the glare, the spot size and focus error. (see Chapter 1 p28) For the estimation of the possible stochastic error repeated measurements of the same nucleus were performed and the following results were obtained.

	No. of measurements	Mean$\pm$S. D.	Standard error	Coefficient of variation
DNA	20	0.65$\pm$0.020	0.004	3.07
Total histones	20	0.90$\pm$0.025	0.005	2.77
Acidic nuclear proteins	20	1.22$\pm$0.033	0.007	2.70
Lysine-rich histones	20	0.64$\pm$0.022	0.005	3.38

d. Muscle histometry Haematoxylin and eosin sections were used. The nuclear areas of at least 100 nuclei, closely apposed to the surface of the transversely sectioned muscle fibre were drawn and measured by planimetry, at a final magnification of x1000. Care was taken to measure only sarcolemmal nuclei. Nuclear volumes were not calculated, since this requires certain assumptions to be made on the irregular shape of the muscle fibre nucleus (Franke and Schinko 1969).

The number of sarcolemmal nuclei of at least 100 transversely sectioned fibres was also estimated and the number of nuclei per muscle fibre computed. The mean muscle fibre size (when needed) was measured by the "lesser fibre diameter" method of Dubowitz and Brooke (1973). The students test (t-test) as well as the "analysis of variance" test were used for the statistical comparison of the various groups.

III. Results

Quantitative histochemistry

The results of this part of the study are summarised in the Table 2.I and Figs. 2.1 and 2.2. As it can be seen, all the parameters examined, namely DNA, histones (arginine- and lysine-rich fractions), and acidic nuclear proteins show

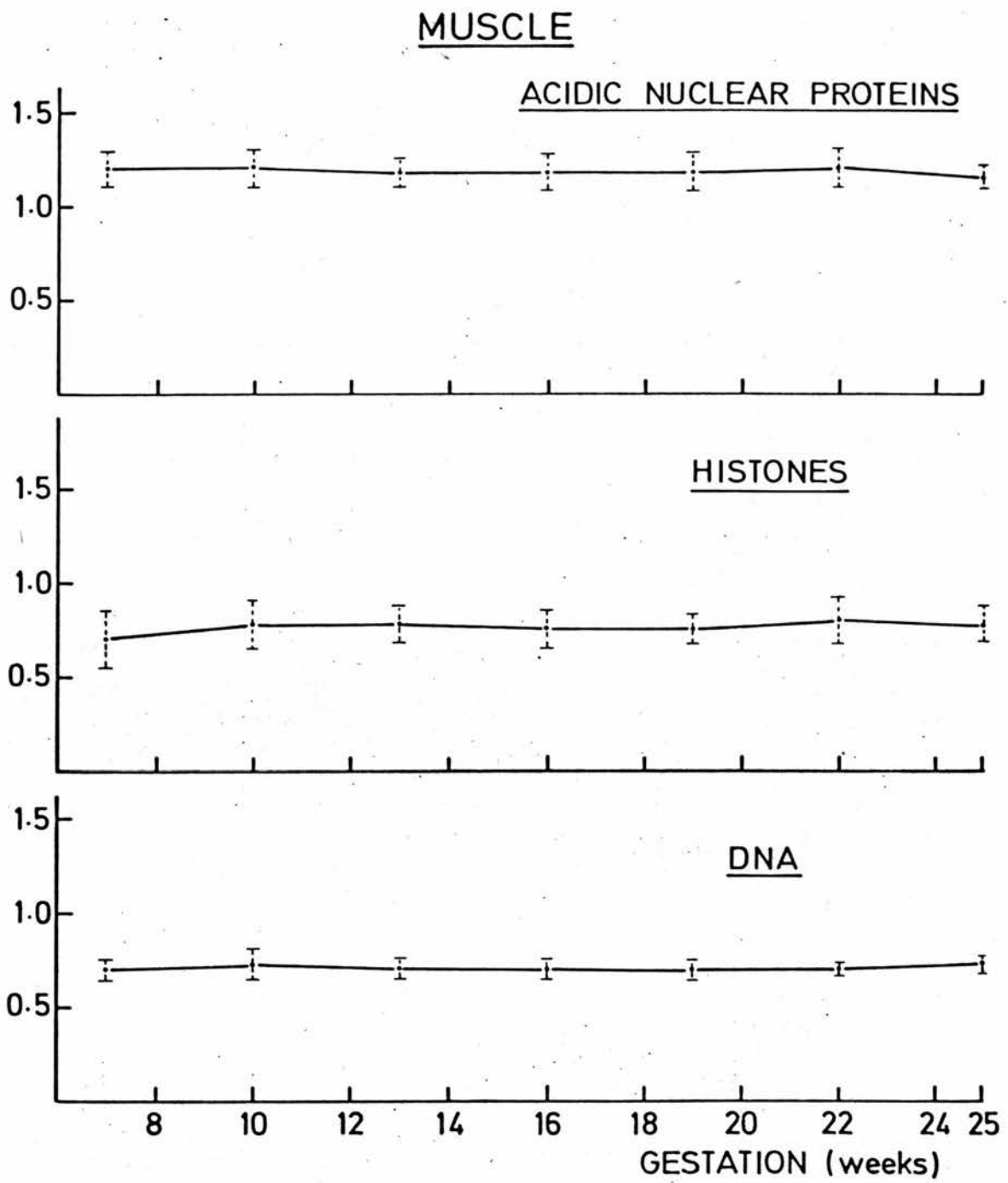


Fig. 2.1 Mean density per unit area of DNA, histones and acidic nuclear proteins in fetal muscle nuclei for different periods of gestation. (The dotted vertical lines represent a standard deviation on either side of the mean value).

Gestation (weeks)	No. of fetuses	D.N.A.	Histones			Acidic Nuclear Proteins
			Arginine-rich	Lysine-rich	Total	
6-8	4	0.70±0.06	0.08±0.06	0.62±0.11	0.70±0.16	1.19±0.09
9-11	6	0.72±0.07	0.09±0.04	0.68±0.11	0.77±0.12	1.21±0.09
12-14	10	0.70±0.03	0.09±0.03	0.67±0.08	0.76±0.10	1.17±0.07
15-17	8	0.71±0.04	0.10±0.03	0.66±0.09	0.76±0.09	1.18±0.10
18-20	6	0.71±0.03	0.07±0.03	0.69±0.07	0.76±0.07	1.18±0.10
21-23	5	0.70±0.02	0.08±0.03	0.71±0.09	0.80±0.12	1.17±0.09
24-26	4	0.72±0.04	0.08±0.03	0.70±0.08	0.79±0.10	1.15±0.08

Table 2.I. Mean density per unit area of DNA, histones and acidic nuclear proteins in fetal muscle cell nuclei for different gestational age groups (Results are expressed as mean ± standard deviation).

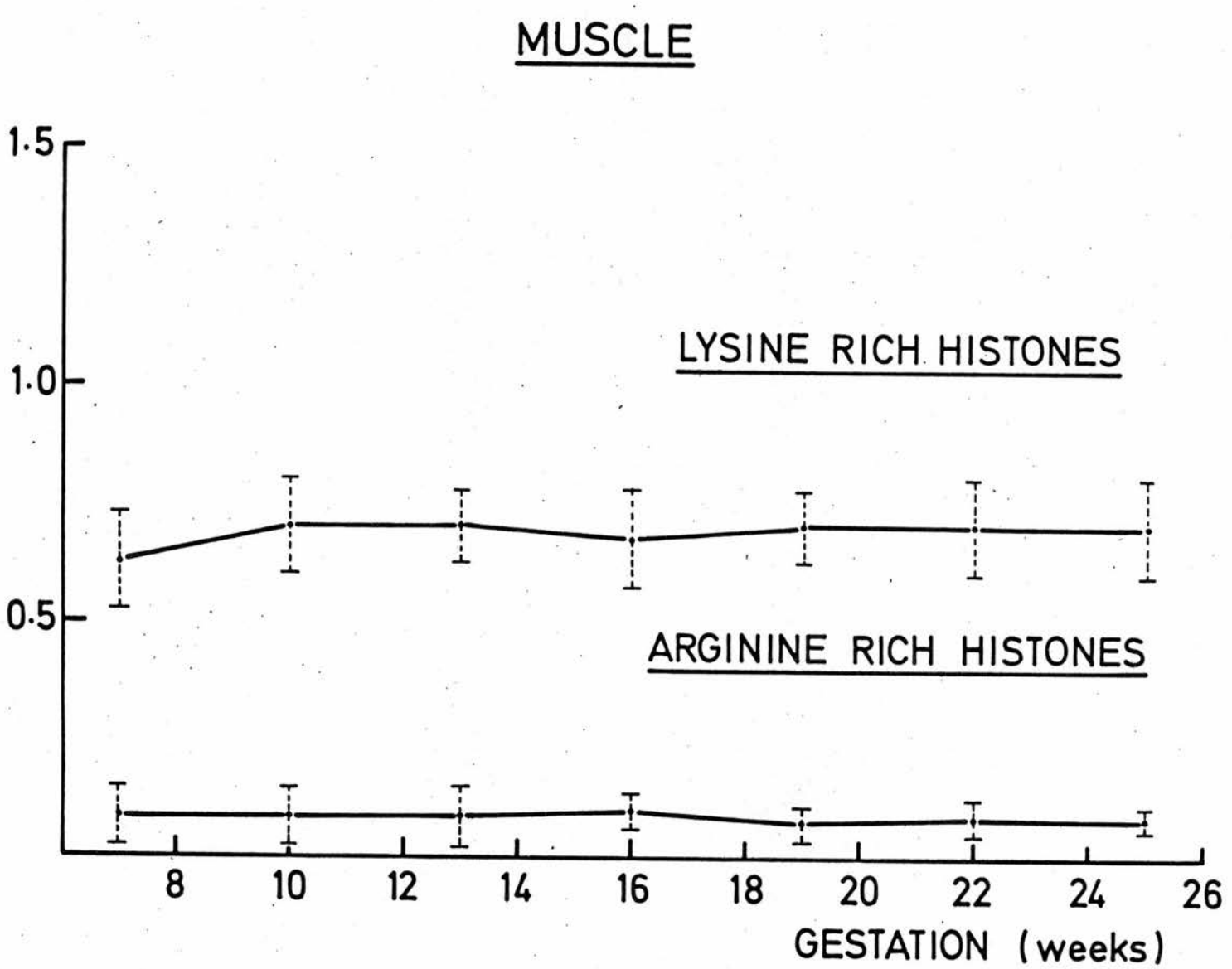


Fig. 2.2 Mean density per unit area of lysine-rich and arginine-rich histones in fetal muscle nuclei for different periods of gestation. (The vertical lines represent a standard deviation).

no marked changes during the gestational period examined.

Muscle histometry

The muscle nuclear size during fetal development is shown in the Table 2.II. and Fig. 2.3.

Gestation (weeks)	No. of fetuses	Nuclear size (μm^2)
6-8	4	22.9 ± 1.4
9-11	6	25.6 ± 3.0
12-14	11	25.4 ± 3.2
15-17	8	27.1 ± 4.6
18-20	6	26.6 ± 2.3
21-23	5	25.5 ± 2.8
24-26	4	25.7 ± 2.9

Table 2.II. Muscle nuclear area for the different gestational age groups (Results are expressed as mean $\pm$ standard deviation).

The muscle nuclear size remains fairly constant during the fetal periods examined. From the same figure (2.3) as well as from the Table 2.III. it can be seen that the muscle

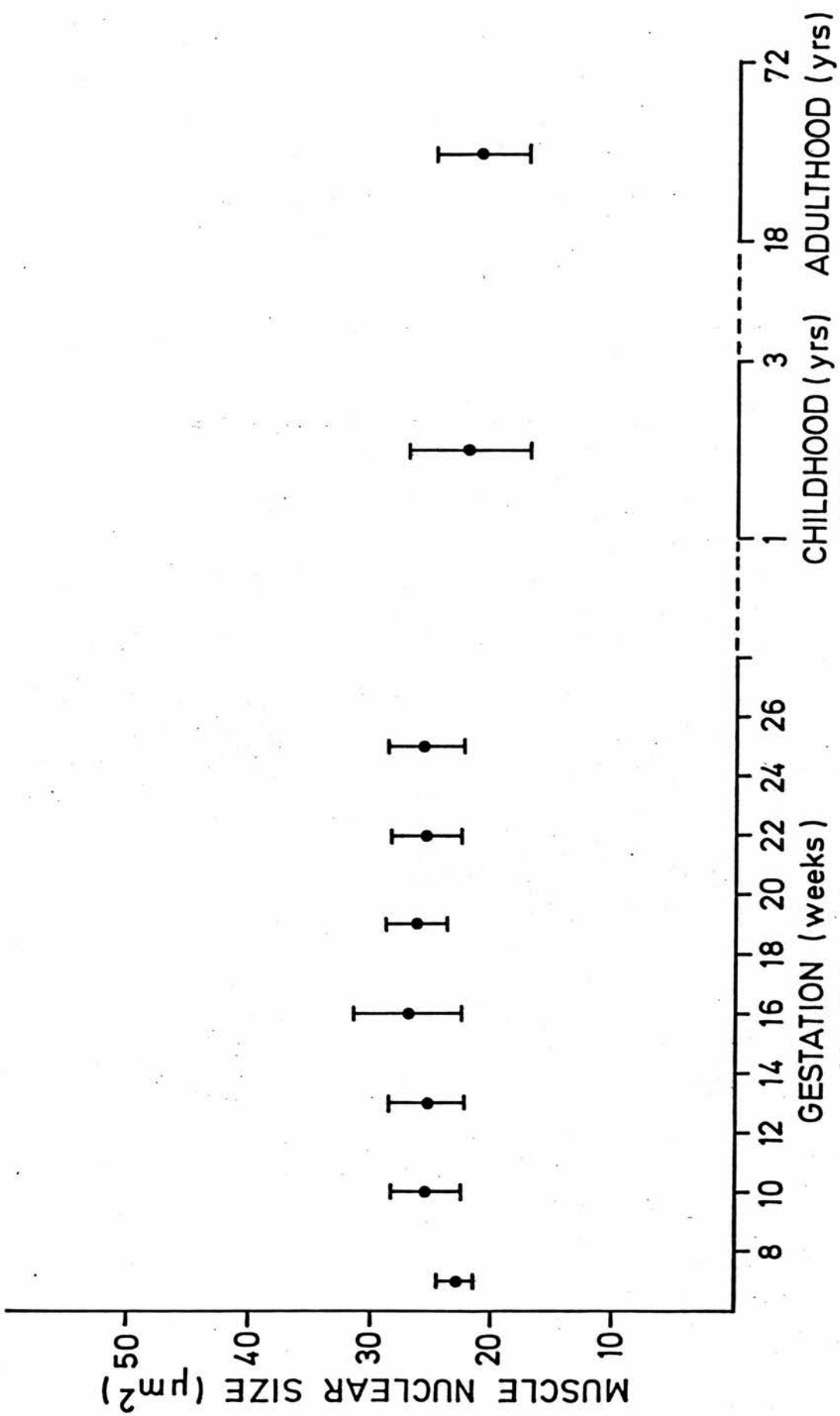


Fig. 2.3 Muscle nuclear size in fetuses of different periods of gestation, juveniles and adults. (The vertical lines represent the standard deviation on either side of the mean value).

	Age	Fibre size (μ m)	No. of nuclei (per fibre)	Nuclear size (μm^2)
Fetal life	8-26 weeks	8.1 ± 1.5	–	25.7 ± 3.2
1st Group	1-2.5 years	21.9 ± 3.1	0.9 ± 0.2	21.8 ± 5.1
2nd Group	12-30 years	53.3 ± 5.1	2.2 ± 0.2	20.8 ± 4.7
3rd Group	32-55 years	55.1 ± 8.3	2.2 ± 0.2	21.3 ± 3.2
4th Group	60-71 years	51.7 ± 8.1	2.5 ± 0.3	21.2 ± 3.8

Table 2.III. Muscle fibre size, number of nuclei and nuclear size in different age groups. (Results are expressed as mean $\pm$ standard deviation).

nuclear size in childhood and adulthood is not appreciably different from that of the fetal muscle. In fact, the muscle nucleus in fetal life seems to be slightly larger than that of the adult. (Plate 2.)

Table 2.III and Fig. 2.4 summarise the observed karyometric changes of the human muscle with age. The number of nuclei per muscle fibre in fetal muscle was not estimated because of the difficulty in distinguishing individual muscle fibres. The first postnatal group examined (age 1-2.5 years) has a much smaller mean fibre size than the other groups in which the mean fibre size does not vary very much. The muscle nuclear size did not appear to vary between the four groups. The observed changes in the mean fibre size were paralleled by the number of nuclei in the first group, but increases dramatically and remains constant among the latter three groups (Fig. 2.4).

Statistical analysis revealed that this value of the number of nuclei per muscle fibre in postnatal life is proportional to the mean muscle fibre size. ($r=0.85$ and $p < 0.001$).

IV. Discussion

It is widely accepted that gene activity causes differentiation even in the early stages of embryonic development.

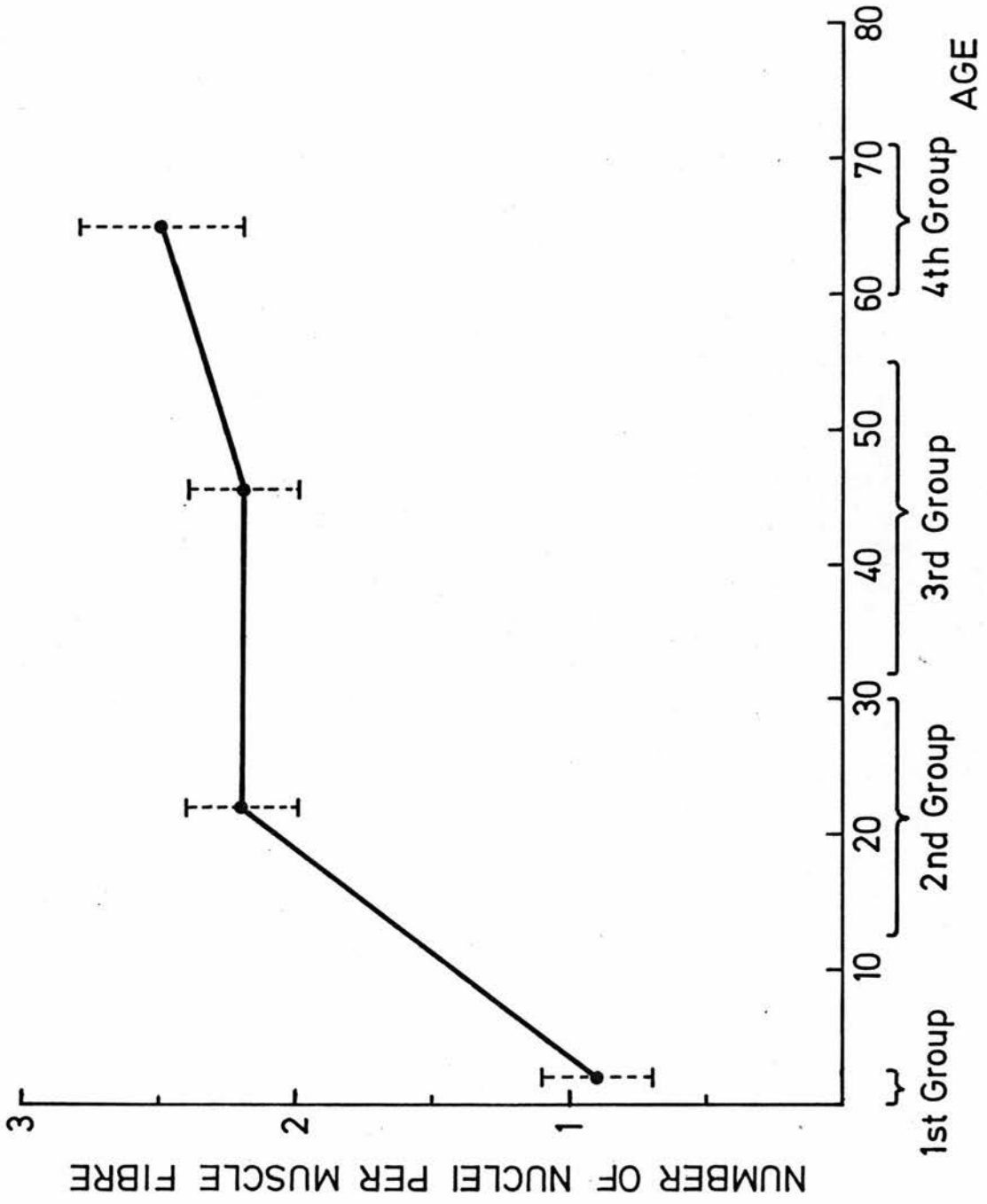


Fig. 2.4 Number of nuclei per muscle fibre cross-section for the various age groups. (The dotted vertical lines represent the standard deviation on either side of the mean value).

Direct connections between various modifiers of development and DNA transcriptions, at a particular genetic locus, has yet to be established.

Genes which code for messenger RNA which results in the formation of specific cell products (e.g. myosin or actin) will be subject to close control by gene regulators. In addition, a similar control of RNA synthesis is expected at the template sites coding for both ribosomal RNA and transfer RNA. Thus, apart from the study of RNA synthesis, it seems that a meaningful approach to the molecular analysis of muscle development is the quantitative assessment of the various gene regulators during development.

These regulators, and acidic nuclear proteins in particular, seem to play an exceptional role in muscle cell function. Acidic nuclear proteins appear to turn over much more rapidly than other muscle proteins and muscle fibre nuclei incorporate aminoacids into acidic nuclear proteins at a very high rate (Schultze and Maurer 1967,, Kuehl 1975). For skeletal muscle, the ratio of nuclear to cytoplasmic incorporation was about 10:1 (Schultze and Maurer 1967). This is in contrast to the situation existing in a wide variety of other tissues in which the incorporation of aminoacids by the nucleus was similar to that of the cytoplasm. It has been suggested, therefore, (Ono and Terayama 1975, Kuehl 1975) that muscle nuclei possess a system of incorporating aminoacids which is different from the ribosomal synthesising system of the cytoplasm.

In skeletal muscle, after the myoblast proliferation has ceased, the development of the contractile system into an extended cross-striated muscle fibre occurs and this requires the fusion of mononucleated myoblasts into elongated, multinucleated myotubes.

At a molecular level, the decline of myoblast proliferation is accompanied by a decrease of the DNA which was required specifically for cell proliferation (Stockdale 1970). It is noteworthy that the activity of enzymes involved in DNA synthesis (DNA polymerase, G-6-PD) is also decreased (Herrmann et al. 1970, Celotti et al. 1973). After fusion, all nuclei, in myotubes, contain a constant amount of DNA (Lash et al. 1957, Firket 1958, Basleer 1962, Cox and Simpson 1970).

In the present study, relatively mature muscle fibres were examined and the finding that the amount of DNA remains constant, during development, was to be expected. During the terminal stages of cell specialization an increase in the concentration of cell specific proteins (contractile proteins of the muscle fibre) is observed. At first, this increase of the contractile proteins starts at a slow rate and reaches a maximum rate of increase later in the muscle cell development. The results of the present study indicate that these changes during the process of muscle cell specialization are not associated with major changes in the regulators of gene activity.

The result of the histometric part of this study confirmed the well documented finding that during childhood the muscle fibre increases dramatically and then remains fairly constant during adult life (Cheek 1968, Brooke and Engel 1969, Sissons 1974). These changes in muscle fibre size are associated with a similar increase in the number of nuclei, a finding which indicates that during normal muscle development, the nucleo cytoplasmic ratio remains virtually constant. This constancy is probably due to an increase in the number of nuclei, the cross-sectional area of which remains constant during not only the whole fetal life, but also the postnatal life. So, in contrast with the motor neurone nucleus which during fetal life is much smaller than the adult one, it seems that the size of the muscle nucleus has already reached its adult size in early fetal life. The immutability of this relationship is illustrated by the strong positive correlation ($p < 0.001$) between the muscle fibre size and the number of nuclei per muscle fibre in the postnatal human.

Enesco and Puddy (1964) described the maintenance of a similar ratio during rat muscle development and MacConnachie et al. (1964) and Moss and Leblond (1971) suggested that the nuclei needed to maintain the nucleo-cytoplasmic ratio constant, come from satellite cell divisions.

The satellite cells do not fuse but remain a separate entity. This, as well as the presence of centrioles in the

satellite cell cytoplasm and absence of them within the multinucleated muscle cell cytoplasm (Muir 1970), suggest that a possible role of the satellite cell is to provide nuclei for the muscle fibre when the nucleo-cytoplasmic ratio is disturbed.

The findings described in this chapter provide a basis for studying changes in the number and size of muscle fibre nuclei (Vassilopoulos et al. 1976a, b, c), but the sequence of events which produce the pathological pattern is at present unknown (see Chapter 5).

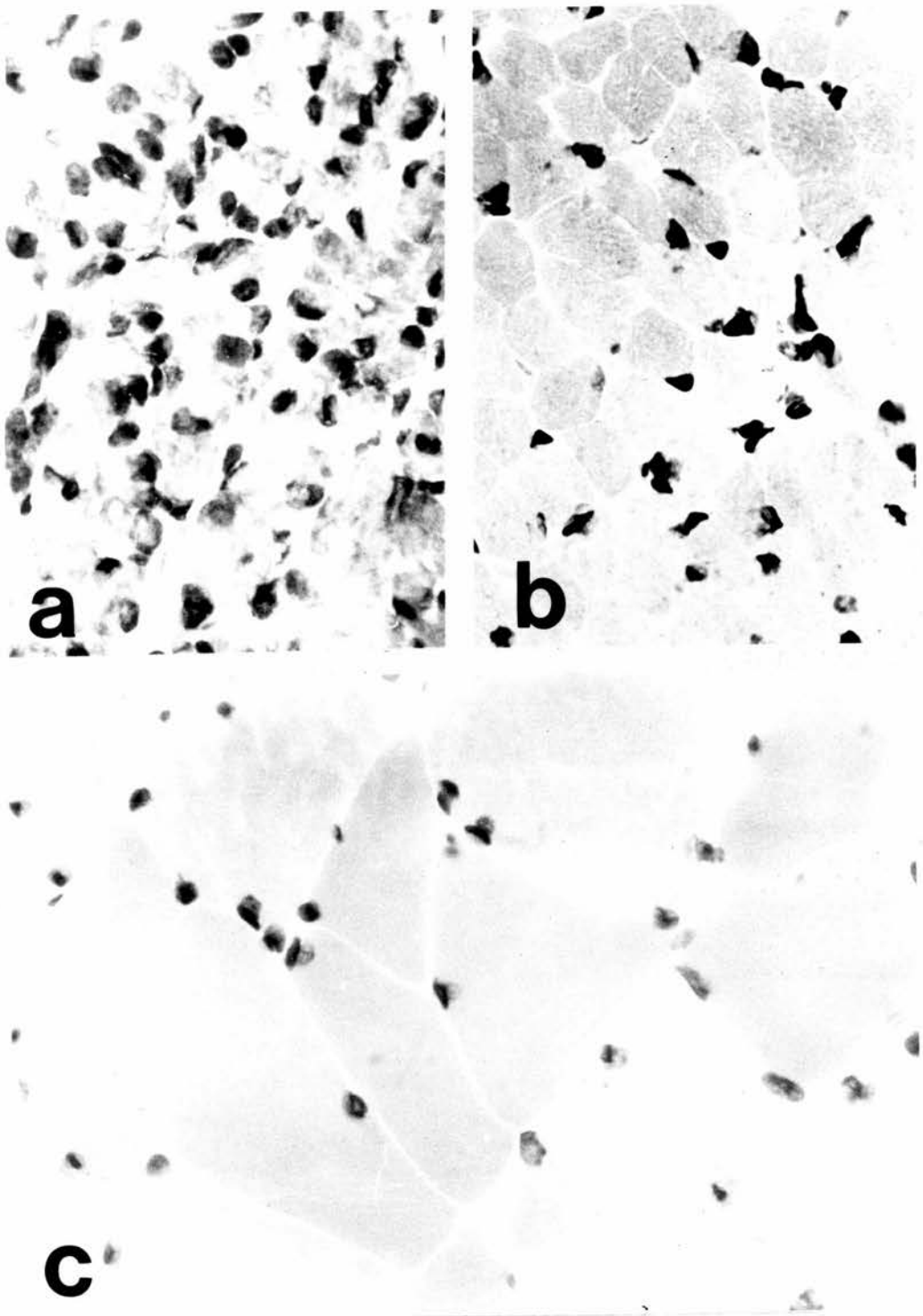


Plate 2. Transversely sectioned muscle fibres of a fetus of 22 weeks of gestation (a), a 1.5 year old child (b) and a 44 year old woman (c). Frozen sections stained with haematoxylin and eosin (x 700).

CHAPTER 3

Nuclear changes in dystrophic muscle in vitro

I. Introduction

II. Material and Methods

i. Material

ii. Methods

III. Results

IV. Discussion

C H A P T E R T H R E E

I. Introduction

Tissue culture studies provide the advantage of examining cells and tissues in isolation, without humoral or neurological influences. Such studies with muscle have given new insight into muscle differentiation, and particularly into the interactions between muscles and the nervous system.

The major steps of myogenesis seem to be well established. Initially, a mononucleated cell population which has prominent mitotic activity accumulates at certain sites destined to form muscle. The next step in myogenesis is the formation of multinucleated syncytia (myotubes) by fusion of the cytoplasm of myoblasts. Synthesis of actin and myosin begins after cell fusion and subsequent development of the

muscle cell involves the co-ordinated assembly of myofibrils and the sarcotubular system, proliferation of mitochondria, deposition of glycogen, and finally, innervation.

The *in vitro* study of myogenesis offers a number of advantages.

1. Living cells are obtained in thin layers (usually only one cell thick) and therefore the morphology of single cells is distinct (Ross *et al.* 1974).

2. Environmental conditions can be easily controlled and it is simple to study the action of various substances on the developing muscle.

3. Myogenesis can be studied in the absence of other cells which might otherwise influence development *in vivo*.

4. Okazaki and Holtzer (1965) have pointed out that in tissue culture the differentiation of muscle cells is more rapid and exhibits a greater degree of synchrony than *in vivo*. The extensive work on human skeletal muscle differentiation *in vitro* has been reviewed by Koningsberg (1965) and Murray (1965, 1972).

Many aspects of muscle development are well documented, but the role of the muscle fibre nucleus (and its relationship to the developing muscle cell cytoplasm) does not appear to have been fully elucidated. Alterations of the nuclear shape, accompanied by chromatin condensation, have been observed during muscle differentiation. (Marchok and Wolff 1968, Fischman 1970). However, Bishop *et al.*

(1971) found no significant differences in the number of nuclei in multinucleated myoblasts or myotubes between normal and diseased muscle.

Ross and his associates (1968, 1974) have studied intensively those intranuclear events occurring during differentiation of myoblasts. They showed (Ross 1964) that nucleoli in binucleated cells have less than one-half the dry mass and volume of those of uninuclear myoblasts. Optical density measurements (Ross et al. 1968) indicated that during fusion, RNA leaves the nucleoli in at least one of the partners at a greater rate than it can be synthesized and appears in unusually high concentration in the cytoplasm. During this process the nucleolus shrinks, to enlarge again after fusion.

Vassilopoulos et al. (1976 a) examining muscle biopsies from spinal muscular atrophies and Duchenne muscular dystrophy (DMD), showed that in DMD the muscle fibre nuclei were enlarged. They concluded that this was just one aspect of a modified nucleo-cytoplasmic relationship (see Chapter 5, page 108). A similar finding (an increase in the muscle nuclear size) was reported in the muscle of 50% fetuses at risk for Duchenne muscular dystrophy. (Vassilopoulos and Emery 1976).

The present study was undertaken to find out if similar changes in the nucleus of muscle fibre occur at the early stages of dystrophic muscle development in vitro.

II. Material and Methods

i Material

Myogenic cell lines were prepared from fresh muscle biopsies of six normal and two dystrophic individuals. The quadriceps femoris was the muscle biopsied and about 4 mm. cubes of tissue were taken.

ii Methods

All lines were obtained and maintained under sterile conditions. The samples were processed as soon as possible after biopsy and were first washed in phosphate buffered saline solution (Dulbecco) containing 100 units/ml. Penicillin, 200 mg./ml. Streptomycin and 2.5 μg/ml. of Amphotericin B (Ca and Mg ion free). This procedure prevented infection occurring in primary cultures. Primary explants were made by dividing the piece of muscle very finely with scalpels and placing approximately 16 small muscle blocks on the inside surface of 100 ml. glass bottles. The explants were clotted using a 1:1 mixture of filtered chick embryo extract and cock plasma for a few minutes. Once clotting had taken place, (usually after a few minutes) the explants were overlaid with Hams F-10 containing 10% fetal calf serum and the same concentrations of antibiotics All media were supplemented with 0.7 mM Calcium (final concentration) which was found to be adequate for the fusion of myoblasts.

The explants were examined microscopically each day

for any evidence of growth and the medium was changed regularly (every 3-4 days), even if no growth was apparent. There was always a delay before growth began, varying from 3-8 days for normal and 8-21 days for dystrophic muscle. Only explants showing well developed myotubes (and hence presumably a considerable myogenic population) were used for secondary cultures. These were set up following Trypsin digestion (using 0.25% Trypsin in Dulbecco (pH 7.4) and washing by centrifugation at 1000 r.p.m. using Dulbecco with antibiotics. Cells were than re-dispersed into larger vessels with medium. When attachment of cells had taken place the medium was changed, thus removing any dead cells or debris.

The cultures were then maintained at 37^{o}C, until a confluent monolayer was obtained. Secondary cultures were observed especially carefully. When sufficient cells were available, and always prior to fusion, the secondary cultures were trypsinised. The serum concentration used was 10%. The differentiation of myoblast was examined at 15 and 25 days on coverslips (with adhering cells) stained with haematoxylin and eosin.

All slides were photographed so that the nuclear area in a random selection of 15 multinucleated myoblasts (Bishop et al. 1971) from each preparation could be measured by planimetry (final magnification of X1000). No measurements of mononucleated myoblasts were taken since these are

essentially indistinguishable from fibroblastic-type cells.

III. Results

The results are summarized in the Table 3.I. It can be seen that at 15 days the nuclear size in the dystrophic multinucleated myoblasts ($24.9 \pm 1.1\ \mu m^2$) is significantly greater ($p < 0.02$) than that of the mormal myoblasts ($22.0 \pm 1.0\ \mu m^2$). At 25 days the difference between dystrophic ($23.8 \pm 0.7\ \mu m^2$) and normal (21.1 ± 1.2) muscle is also statistically significant ($p < 0.05$). (Plate 3).

IV. Discussion

The use of muscle cell culture has, in the last few years, considerably extended our knowledge of the various aspects of cell differentiation. Although skeletal muscle from patients with muscular dystrophy exhibits striking histological changes, the *in vitro* study of its development has failed to detect major differences from normal. Several investigators have reported certain morphological differences between normal and dystrophic muscle *in vitro*, but their results show a considerable degree of contradiction. For example, O'Steen (1963) found that in cultures of dystrophic muscle the size of mononucleate myoblasts was similar to that of the normal muscle cultures, while the multinucleate myoblasts were larger

	Identification	Fetal calf serum concentration	Nuclear size (in μm^2) 15 days	25 days
Controls	S. D.	10%	21.8 ± 3.1	19.9 ± 2.6
	N. J.	10%	22.4 ± 2.9	23.0 ± 2.9
	C. R.	10%	23.8 ± 3.0	20.3 ± 2.9
	W. D.	10%	21.9 ± 2.6	20.8 ± 2.7
	W. C.	10%	21.2 ± 2.3	22.5 ± 3.0
	A. M.	10%	20.9 ± 1.9	20.5 ± 2.2
Dystrophic	A. G.	10%	25.7 ± 3.2	23.3 ± 2.9
	A. W.	10%	24.1 ± 3.0	24.3 ± 2.8

Table 3.I. Nuclear size in multinucleated myoblasts from normal and dystrophic muscle cultured in vitro. (The results are expressed as mean area $\pm$ standard deviation).

and the myotubes shorter than in the controls. On the other hand, Goyle et al. (1967) reported that myoblasts from dystrophic muscle were smaller and more variable in size and shape, and that there were fewer multinucleate cells. Kakulas et al. (1968) observed irregular multipolar myoblasts in cultures from dystrophic muscle and slower myotube formation than in the control muscle. Changes in cross-striation in dystrophic muscle studied in vitro have also been reported by several workers. (Geiger and Garrin 1957, O'Steen 1963, Goyle et al. 1968).

Bishop et al. (1971) reported that normal and diseased human muscle had both been successfully grown in tissue culture and had differentiated to myotubes with development of cross-striation. They reported that the length and breadth as well as the number of nuclei in dystrophic myoblasts were not significantly different from those in normal muscle. In an attempt to explain the similarity in the morphology between normal and dystrophic muscle in culture, the authors suggested that some environmental or neural factor might be responsible for the in vivo differences. Another explanation is that the myoblasts from dystrophic muscle are, in fact, abnormal, but to an extent which does not show up in basic morphology, or the length of survival in culture. Our results show that the nuclear size in dystrophic myoblasts is significantly greater than that of the normal myoblast cultured under the same conditions. This morphological difference between

normal and diseased muscle would not be easily detectable by qualitative techniques as used by Bishop et al. (1971).

Nuclear enlargement seems to reflect the histological features observed in the muscle of patients with Duchenne muscular dystrophy or of fetuses at risk. (Vassilopoulos et al. 1976 a, Vassilopoulos and Emery 1976). This finding may reflect a modified nucleo-cytoplasmic relationship which could be due to molecular exchanges between cytoplasm and nucleus. Considerable progress has been made in identifying these chemical changes, but unfortunately much slower progress is being made in explaining the biological effects on developing cells. Speculation has been limited to two general areas: (1) regulation of gene activity and (2) transport of gene products. Enlargement of nuclei is usually paralleled by movement of certain molecules into the nucleus and often accompanies a general change in nuclear activity (Lewin 1974, Goldstein 1974).

The exact role of the nucleus in muscle fibre function is not yet fully understood. The genetic nature of some heriditary neuromuscular disorders suggests some nuclear involvement, but Peterson (1974) reported that nucleus may not have an important function in the dystrophic process. Peterson's technique of chimaera formation from normal and dystrophic morulae is of considerable interest. Using isoenzymes to follow dystrophic and normal nuclei he found that some skeletal muscle fibres had only dystrophic nuclei.

Despite this, these skeletal muscles showed little or no pathological degeneration. Conversely, some of the chimaera muscles contained normal nuclei and yet showed pathological changes. Peterson explained these results by suggesting that at least in these mice the muscle degeneration is caused by an abnormality of extramuscular origin. In any case, our results suggest that the increase in nuclear size observed in dystrophic muscle is present even at the early stages of muscle cell differentiation.

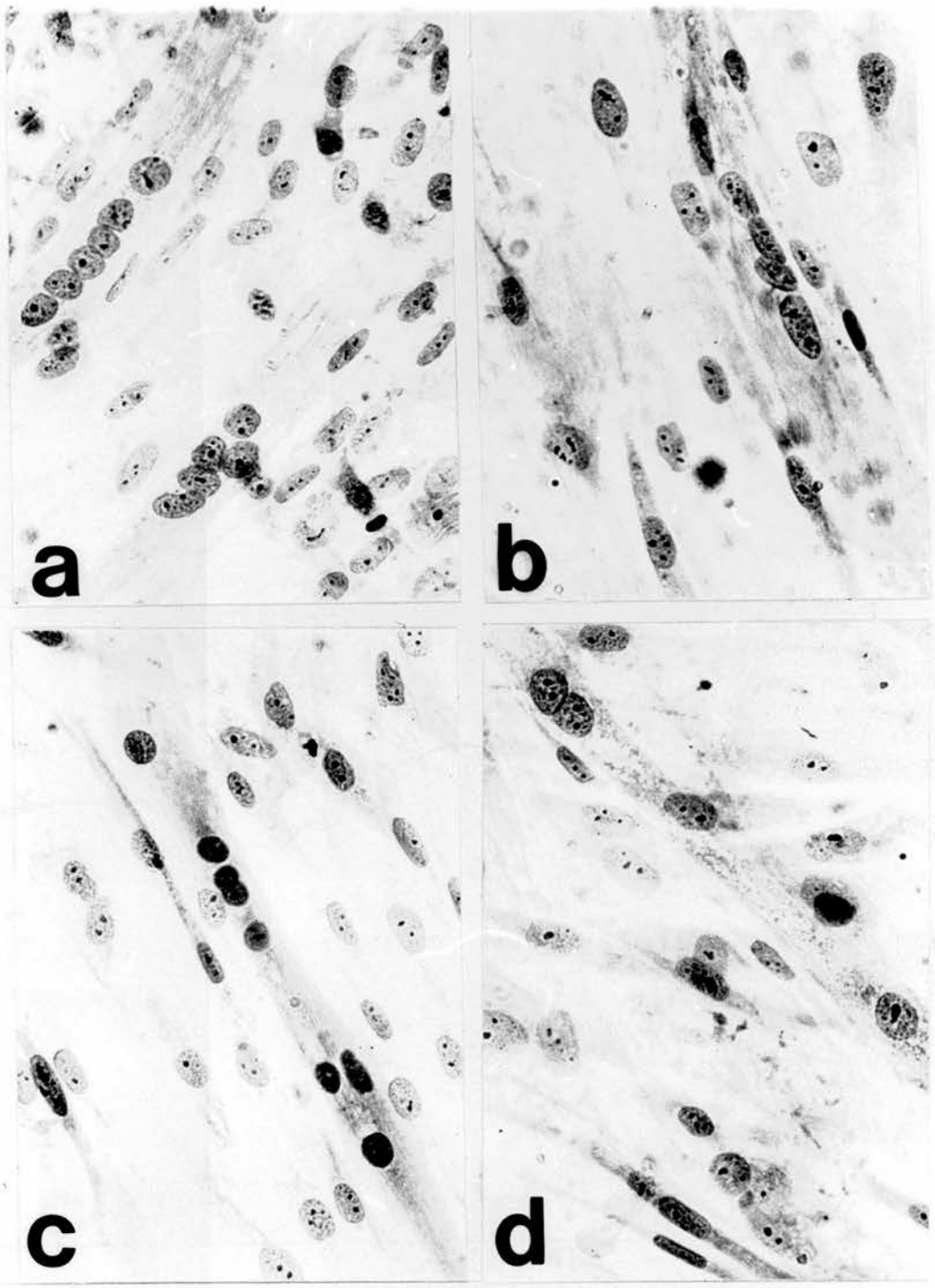


Plate 3. Multinucleated myoblasts from (a) normal and (b) dystrophic muscle after 15 days in subculture and from (c) normal and (d) dystrophic muscle after 25 days in subculture. Haematoxylin and eosin (x 450).

CHAPTER 4

Muscle nuclear changes in fetuses at risk for Duchenne muscular dystrophy

I. Introduction

II. Material and Methods

- i Material
- ii Methods

III. Results

IV. Discussion

CHAPTER FOUR

I. Introduction

The X-linked Duchenne form of muscular dystrophy is the most severe one. The first manifestation of this disease is a clumsiness in walking with a tendency to fall, and muscle weakness. Although there is a considerable variation in the age of onset, these symptoms usually become evident about the age of 3 to 5 years.

The muscle pathology and the increased levels of enzymes released from muscle in cases of Duchenne muscular dystrophy are well documented. There is good evidence from both muscle histology and histochemistry (Pearson 1962, Hudgson et al. 1967, Bradley et al. 1972) and serum enzyme studies (Pearson 1957, Fowler and Pearson 1964, Pearce et al. 1964, Heyck et al. 1966

Bradley et al. 1972) to support that the pathological process may start much earlier than the clinical manifestations of the disease. Toop and Emery (1974) reported changes in muscle histology, similar to those described in preclinical cases, in fetuses at risk for Duchenne muscular dystrophy, and Webb (1974) suggested that a derangement in the normal process of muscle cell death could explain the pathogenesis of the muscular dystrophies.

Recently, Vassilopoulos et al. (1976) reported a significant increase in muscle nuclear size in cases of Duchenne muscular dystrophy. This finding was explained as being probably a reflection of alterations in muscle fibre nucleo-cytoplasmic relationships.

The present study was undertaken in order to determine if similar changes are also present in fetal dystrophic muscle. The results of this study have been published elsewhere.

II. Material and Methods

i Material

Muscle tissue from eight male fetuses at risk for Duchenne muscular dystrophy was examined. In four of these fetuses (No. 979, No. 1018, No. 75/479 and No. 76/60) the mothers were definite carriers and in the remaining four cases the mothers were possible carriers, but at high risk (greater than 1 in 10) of having an affected son. In each case the sex of the fetus was established prior to abortion by sex chromatin and fluorescent studies on uncultured amniotic fluid cells and from karyotype analysis of cultured amniotic fluid cells. The results were compared with the findings in eight male fetuses of comparable gestational age obtained at abortion performed for social reasons and where there was no history of any neuromuscular disorder. The gestational age of the fetuses was estimated by crown-rump (Hamilton and Boyd 1962) and heel-toe (Streeter 1921) measurements.

ii Methods

The quadriceps femoris muscle was chosen for this investigation since it is one of the first muscles to become affected (Walton and Gardner-Medwin 1974). Small blocks of muscle were frozen in isopentane chilled with liquid nitrogen (see Chapter I). Haematoxylin and eosin stained transverse sections 10μ thick were examined. The cross-sectional areas of at least 100 nuclei closely apposed to the surface of transversely sectioned muscle fibres were estimated by planimetry at a final magnification

of x 1000. The nuclei were selected for measurement in this way in order to eliminate fibroblasts, pericytes, endothelial cell and satellite cell nuclei. Nuclear volume was not estimated since this requires certain assumptions to be made regarding the irregular shape of the muscle nucleus (Franke and Schinko, 1969). All measurements were made 'blind' i.e. without any knowledge as to the source of the material.

III. Results

The results of the present study are given in the Table 4.I. Although there is overlap in the individual results obtained, the overall mean size of muscle nuclei in the group at risk for Duchenne muscular dystrophy (35.0 μm^{2+} 4.2) is greater than in the matched controls (28.9 μm^{2+} 2.9), the difference being statistically significant ($p < 0.002$).

It is not known whether the fetuses at risk are going to develop Duchenne muscular dystrophy, but a proportion of them will, since they come from carrier mothers. The figure observed

	Fetus No.	Mother's Status	Gestational age (weeks)	Nuclear size (μm^2) (mean±SD)
Controls	837	N	16	32.0±8.3
	73/125	N	21	34.1±8.4
	73/101	N	22	29.9±7.2
	73/130	N	18	27.2±5.6
	2105	N	19	29.2±6.4
	73/127	N	22	27.8±5.6
	909	N	16	24.9±4.8
	72/22	N	22	26.8±4.9
Fetuses at risk	979	D	20	33.2±8.4
	1018	D	21	34.1±7.8
	75/479	D	18	28.1±7.3
	76/60	D	22	39.8±8.4
	684	P	16	31.1±8.8
	734	P	16	40.8±9.3
	73/375	P	19	35.9±7.7
	599	P	21	37.1±11.6

Table 4.I. Muscle nuclear size in normal fetuses and in fetuses at risk for Duchenne muscular dystrophy (N=normal, D=definite carrier, P=possible carrier)

in this study, showed no evidence of bimodality and surprisingly the lower values of fetuses at risk fall into the upper half of the control values. However, considering the high variability of the muscle fibre nuclear size (see Chapter 2), the probability that this is due purely to chance is not very low.

IV. Discussion

Although changes in muscle of fetuses at risk for Duchenne muscular dystrophy have been reported, the earliest histologic manifestation of the disease is still unknown, and there is considerable doubt if dystrophic muscle has at any time a normal histological appearance. Pearson (1962) examined a muscle biopsy of a two month old boy with preclinical Duchenne muscular dystrophy and observed widespread hyalinization of the muscle fibres and an increased variation in their size. In addition, in this biopsy there was a moderate increase in the amount of interstitial connective tissue and some increase in basophilia of many fibres which were shown to contain significantly increased amounts of RNA. Bradley et al. (1972) in a muscle biopsy from a $2\frac{1}{2}$ week old boy later

developing Duchenne muscular dystrophy with the full spectrum of histological and histochemical changes characteristic of the disease, confirmed Pearson's observations and pointed out that pathological changes might well be evident even earlier.

In a study of muscle histology in fetuses at risk for Duchenne muscular dystrophy, Toop and Emery (1974), in fact, reported abnormalities similar to those described in preclinical cases (presence of hyaline fibres, increased variability of muscle fibre diameter, increase in the amount of interstitial connective tissue). The authors pointed out that the observed hyaline fibres were neither the result of autolysis nor cells undergoing some form of autolytic degradation which has been shown to occur in the course of the normal myogenesis (Webb 1972), and they concluded that certain histological changes are already evident in utero.

In the present study the observed enlargement of muscle nuclei in fetuses at risk for Duchenne muscular dystrophy and the increase in muscle nuclear size in patients with Duchenne muscular dystrophy (Vassilopoulos et al. 1976) probably reflect the same underlying pathogenic process. (Plate 4).

Nuclear changes in a variety of tissues have been reported in a number of diseases and experimental conditions but the significance of such findings is still controversial (Heiberg 1957, Guimarães 1971). Regarding muscle fibre nuclei it has been suggested that their size is variously related to cellular hypertrophy (Doljanski 1960, Goss 1964), altered ionic environment (Davies and

Spencer 1962) and the degree of muscle contraction (Franke and Schinko 1969). Enlargement of the nucleus seems to be one of the earliest and most consistent responses to alterations in nuclear environment. Much information concerning nucleo-cytoplasmic relationships has been gained from nuclear transplantation experiments in amphibia (Gurdon 1968, Gurdon 1970) and changes in nuclear size may well be related to alterations in gene expression (Lewin 1974). Nuclear enlargement seems to be essential for derepression of the genome of differentiated cells after either nuclear transplantation (Graham et al. 1966, Gurdon 1968, 1970) or somatic cell fusions (Harris 1970, Barry and Merriam 1972). This enlargement depends upon the specific uptake of proteins from the cytoplasm (Arms 1968, Merriam 1969, Goldstein 1974) which enter to the injected nuclei even before there are any signs of enlargement (Lewin 1974). This suggests that this uptake is not a consequence of the enlargement but it may be needed for gene activation. The protein entering the nucleus add to, rather than replace, those previously present (Gurdon 1970). The increase in nuclear volume is paralled by synthesis of RNA (Gurdon 1968) and nuclei which fail to enlarge do not incorporate label into DNA and cannot support development. The changes in nuclear size, in transplantation experiments, are accompanied by morphological changes in chromatin (Graham et al. 1966, Gurdon 1968, Lewin 1974) and in Gurdon's words "Nuclear swelling does not itself induce any kind of nuclear change, but should rather be regarded as a process of derepression, the result of which is to make chromosomes more reactive to the

particular cytoplasmic environment in which they happen to lie".

The observed changes in muscle nuclear size in Duchenne muscular dystrophy and in fetuses at risk for Duchenne muscular dystrophy could be explained as a non-specific nuclear response to altered cytoplasmic environment of the muscle fibre. In any event the finding reported, in this study, provides further support for Toop and Emery's suggestion that Duchenne muscular dystrophy is already manifest in utero by the second trimester of pregnancy.

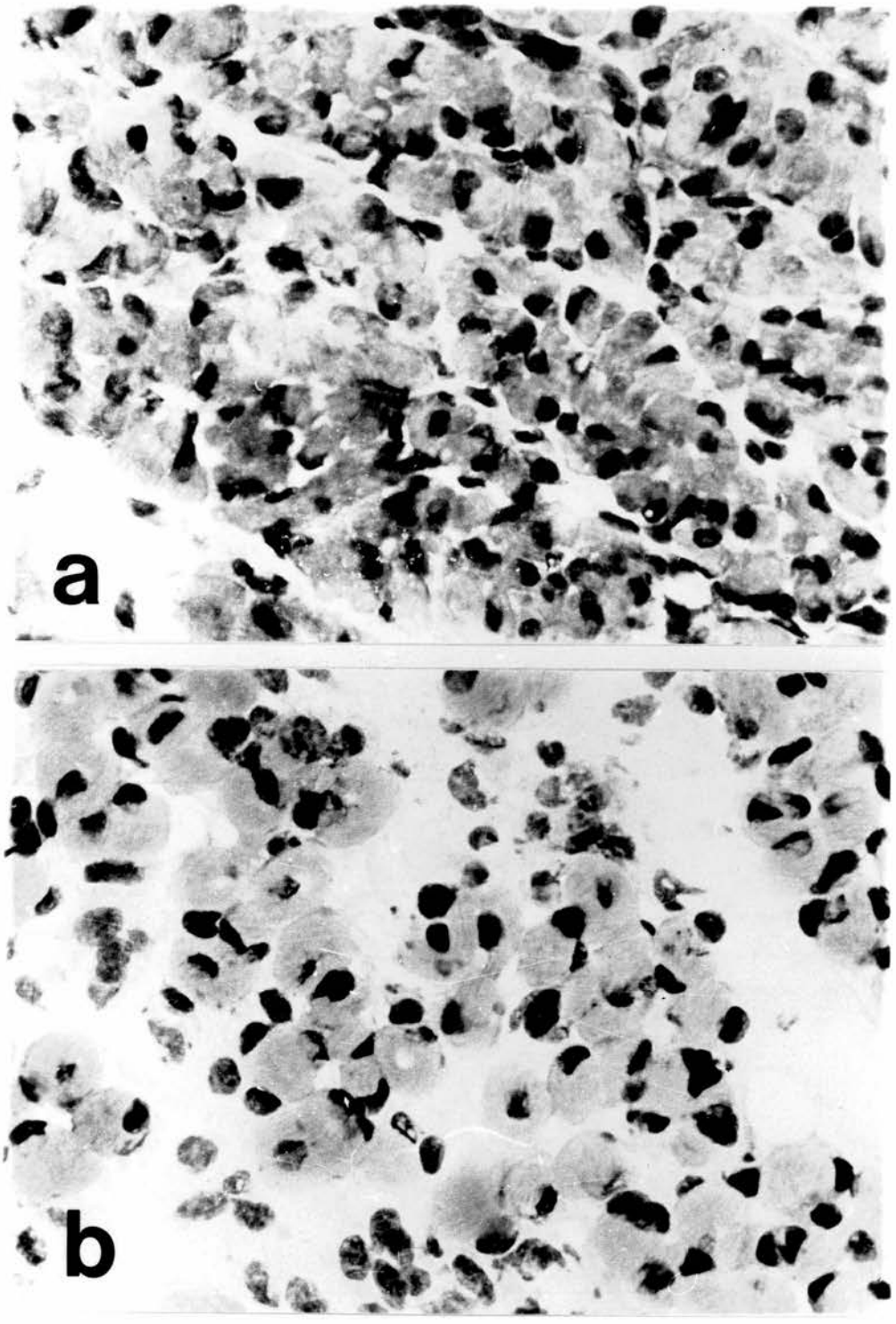


Plate 4. Transverse section of muscle in a normal fetus of 22 weeks of gestation (a) and in a male fetus at risk for Duchenne muscular dystrophy (b) of the same gestational age. Cryostat sections stained with haematoxylin and eosin (x 700).

CHAPTER 5

Muscle nuclear changes

in disease

I. Introduction

II. Material and Methods

i Material

1. Childhood neuromuscular disease.
2. Chronic neuromuscular disease.
3. Diabetic neuropathy.
4. Myotonic dystrophy.

ii Methods

III. Results

1. Childhood neuromuscular disease.
2. Chronic neuromuscular disease.
3. Diabetic neuropathy.
4. Myotonic dystrophy.

IV. Discussion

CHAPTER FIVE

I. Introduction

The vast amount of research into the neuromuscular diseases has, as yet, failed to reveal their pathogenesis. The histological and histochemical alterations of the muscle fibres in these diseases have been well documented, but little attention has been paid to the muscle cell nucleus. Although at the moment it is impossible to detect the primary single gene defect for each hereditary neuromuscular disease, a histometric examination of the nucleus may indicate the extent of its participation in the pathological process.

The variable nature of the muscle nucleus is

demonstrated during normal postnatal muscle development when an increase in the size and number of muscle fibres (Cheek 1968). A similar increase in size and/or number of nuclei would be expected in order to keep the nucleo-cytoplasmic ratio within certain limits.

An increase in nuclear size in other cell types occurs in experimental animals in conditions such as cellular hypertrophy after partial ablation of the liver (Stowell 1948, Hammarsten 1951, Harkness 1952, Wilson et al. 1953) or kidney (Sulkin 1949, Schmiedt 1951, Fajers 1957). Enlargement of cell nuclei has also been observed in the cirrhotic liver (Guimarães 1971) as well as in the thyroid gland (Alfert et al. 1955) and hypothalamus (Bandaranayake 1974) under experimental conditions.

As mentioned previously (Chapter 2, page 52) striated muscle is an exceptional tissue being a syncytium. There is evidence that in the developing muscle, the maintenance of the nucleo-cytoplasmic ratio is obtained by an increase in the number of nuclei per muscle fibre (Enesco and Puddy 1964, Vassilopoulos et al. 1976c).

In diseased muscle, the apthological process may be reflected in an altered nucleo-cytoplasmic ratio. The elucidation of possible changes in this ratio would be helpful in understanding the nature of the muscle's response to various disease processes.

In an attempt to study the very wide spectrum of

neuromuscular diseases, four groups of characteristic neuromuscular involvement were studied. The first group consisted of Duchenne muscular dystrophy and spinal muscular atrophies, i.e. the childhood neuromuscular diseases. In the second group adult neurogenic atrophies and muscular dystrophies were examined. In the third group diabetic neuropathy was chosen, being a characteristic chronic demyelinating neuropathy. Finally, myotonic dystrophy, a disease the purely myopathic nature of which is in question, was studied.

In the first two groups, nuclear changes do not seem to have been reported and the aim of the present study was to detect possible involvement of the nucleus in denervating and dystrophic processes. In the latter two groups (diabetic neuropathy and myotonic dystrophy) changes in muscle nuclei have been reported (Locke et al. 1963, Bloodworth and Epstein 1967, Dubowitz and Brooke 1973, Adams 1974, Malene and Stroia 1974), but they lack accurate quantitation. It is generally considered that after differentiation myonuclei do not undergo mitosis, so in addition to a quantitative assessment, the aim of the study of these diseases was to determine the nature of possible changes in nuclear number.

II. Material and Methods

i Material

The material for this study consists of four groups of patients suffering from various diseases involving the neuromuscular system. In these groups the muscle nuclear size and/or the number of nuclei per muscle fibre were calculated.

Group 1. Childhood neuromuscular disease. In this part of the study the material used consisted of 7 controls (mean age 2.4 years), 8 patients with type I (Werdnig-Hoffmann) and type II spinal muscular atrophy (Fried and Emery, 1971) (mean age 2.8 years) and 8 patients with Duchenne muscular dystrophy. The mean duration of illness was 0.8 years for the spinal muscular atrophies and 3.0 for Duchenne muscular dystrophy. The age and sex of the individuals are shown in Table 5.I. In almost all the cases the vastus lateralis was the muscle biopsied. The number of nuclei was not estimated because of the difficulty in estimating the number of nuclei in the small atrophic fibres.

Group 2. Chronic neuromuscular disease. Muscle biopsies from 10 adult patients (5 males and 5 females) with different forms of chronic neurogenic atrophy and 10 adult patients (6 males and 4 females) suffering from chronic myopathy were examined. Five out of the 10 patients with neurogenic atrophies suffered from Scapuloperoneal S. M. A., 3 from adult S. M. A. and 3 from various other forms of S. M. A.

Half of the patients with myopathy suffered from fascio-scapulohumeral muscular dystrophy and the other half from limb girdle muscular dystrophy. The mean age of the patients was 32.4 years for the neurogenic atrophies and 31.9 years for the myopathies. The mean duration of illness was 4.6 years for the neurogenic atrophies and 6.1 years for the myopathies. The quadriceps femoris or the vastus lateralis muscle was biospied.

Group 3. Diabetic neuropathy. Muscle biopsies from 8 controls (4 males and 4 females, aged 26 to 69 years) and 8 patients suffering from diabetic neuropathy (4 males and 4 females, aged 52 to 75 years) was the material used. The mean duration of illness was 3.3 years for the diabetes and 1.0 years for the neuropathy. For this study, the quadriceps femoris was chosen for biopsy under general anaesthesia.

Group 4. Myotonic dystrophy. Eight patients with myotonic dystrophy (3 males and 5 females) and 8 age and sex matched controls were used for this study. The mean age was 39.1 years for the myotonic and 38.9 years for the controls. The mean duration of illness of the myotonic patients was 14.5 years. The quadriceps femoris was also the muscle studied. Details of the individuals are shown in the Table 5.V.

ii Methods

In all cases the diagnosis was based on clinical examination and confirmed by biochemical (serum creatine kinase levels)

and electrophysiological (electromyogram, nerve conduction velocity) studies as well as by standard muscle histology. The piece of muscle was frozen in isopentane chilled in liquid nitrogen. Sections 10 μm thick were cut on a "Slee" cryostat and attached to glass slides, at room temperature. Haematoxylin and eosin stained sections were used throughout this study and the readings were made blindly.

For the studies with spinal muscular atrophies and Duchenne muscular dystrophy, four randomly chosen fields were photographed, the negatives were enlarged to a final magnification of X1000 and the area of at least 100 nuclei closely apposed to the surface of the transversely sectioned muscle fibre was measured by planimetry. In this study only nuclear area was estimated since any estimation of the muscle nucleus volume requires certain assumptions, possibly invalid, to be made on the shape of the muscle nucleus (Franke and Schinko 1969).

In the other patients studied (groups II, III and IV), the number of sarcolemmal nuclei apposed to 100 transversely sectioned muscle fibres in 6 random fields was estimated and the number of nuclei per muscle fibre computed.

The nuclei were selected for measurement in this way in order to eliminate fibroblast, pericyte, endothelial cell and satellite cell nuclei. In many cases for precise identification of the sarcolemmal nuclei, ultra thin sections of Spurr-embedded muscle were stained with lead citrate and uranyl-

acetate and examined in an AE1 6B electron microscope. (Plate 5B). The student's test (t-test) was used for a statistical analysis of the results.

III. Results

The results of this study are as follows:

Group 1. Childhood neuromuscular disease. From Table 5.I. it can be seen that there is an overlap of individual values between the three groups studied, but the overall mean for nuclei in patients with Duchenne muscular dystrophy ($29.73 \pm 4.33\ \mu m^2$) is significantly greater ($p < 0.02$) compared with controls ($23.01 \pm 6.22\ \mu m^2$). There was no significant difference between the values in patients with spinal muscular atrophy and control subjects. (Plate 5.A).

The observed differences could not be correlated with the patient's age or sex. In addition, in the dystrophic group there was no correlation between the nuclear area and various clinical parameters (for example, duration of illness, serum creatine kinase levels etc.).

Group 2. Chronic neuromuscular diseases Tables 5.II, 5.III. and Fig. 5.1. summarise the results of this study. The mean number of nuclei per muscle fibre in the chronic neurogenic atrophies (3.34 ± 0.80) is higher than that of the muscular dystrophies (2.10 ± 0.28). This difference is statistically significant ($p < 0.001$). No differences in muscle

Group	Identification	Sex	Age (years)	Nuclear area (μm^2)
Controls	B84	M	1	22.25 ± 7.03
	138348	F	2.5	29.57 ± 9.61
	144063	F	1.5	29.39 ± 11.50
	B23	M	6	22.14 ± 8.96
	B52	M	2.5	14.81 ± 5.11
	B53	M	1.5	20.74 ± 7.08
	B51	M	2	22.20 ± 6.76
Spinal Muscular Atrophies	8223	M	6	14.21 ± 3.87
	J.Q.	M	6	31.49 ± 9.26
	B48	F	1	22.36 ± 7.80
	131590	M	3	21.54 ± 7.55
	B62	F	0.5	22.22 ± 5.99
	B32	M	0.5	30.94 ± 9.96
	B10	F	4	15.65 ± 4.37
	B72	M	1.5	20.86 ± 8.19
Duchenne Muscular Dystrophy	B70	M	5.5	28.94 ± 10.21
	B65	M	5	33.12 ± 14.04
	B73	M	3.5	31.02 ± 8.57
	B28	M	6	27.87 ± 8.16
	B63	M	4.5	22.60 ± 7.87
	B36	M	2	25.41 ± 7.16
	B34	M	5.5	34.89 ± 13.85
	B47	M	6	34.02 ± 12.84

Table 5.I. Nuclear area in biopsy specimens from Controls and patients with Spinal muscular atrophy and Duchenne muscular dystrophy. (Results expressed as mean ± S.D.)

	Identification	Age (years)	Sex	Number of nuclei (per fibre)
Neurogenic atrophies	B.O.	20	M	3.93
	A.N.	65	M	2.93
	R.S.	19	M	4.16
	J.C.	28	M	3.25
	M.W.	13	F	2.70
	A.H.	54	F	2.24
	M.G.	32	F	3.46
	E.T.	27	F	4.18
	V.I.	35	F	2.24
	K.B.	31	M	4.39
Muscular dystrophies	J.E.	13	F	2.05
	D.T.	21	M	2.09
	P.S.	22	F	2.79
	B.L.	33	M	2.17
	K.L.	28	M	2.08
	E.G.	16	M	1.88
	J.E.	58	M	2.04
	M.S.	59	M	1.79
	M.E.	40	F	1.82
	M.O.	29	F	2.29

Table 5.II. Number of sarcolemmal nuclei per muscle fibre in biopsies from adult neurogenic atrophies and muscular dystrophies.

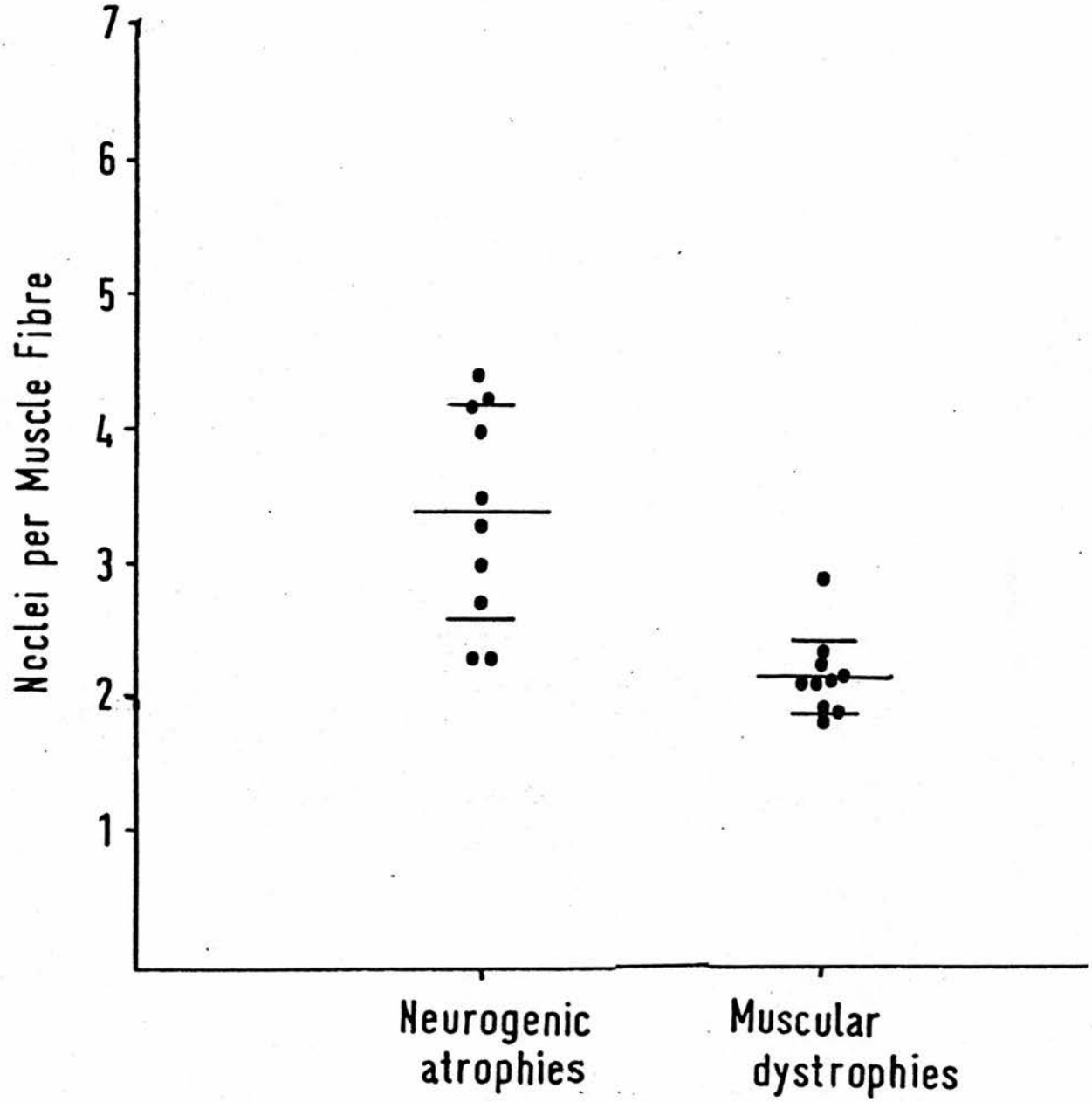


Figure 5.1. Mean number of nuclei per muscle fibre in patients with neurogenic atrophies and and muscular dystrophies.

nuclear size were detected between the two groups studied ($p > 0.10$).

	Age	Sex	Nuclear size (μm^2)	Number of nuclei per fibre
Neurogenic atrophies	32.4	5M 5F	24.4 ± 2.3	3.34 ± 0.80
Muscular dystrophies	31.9	6M 4F	23.4 ± 3.7	2.10 ± 0.28

Table 5.III. Muscle nuclear size and number of nuclei per muscle fibre in patients with neurogenic atrophy and muscular dystrophies. (The results are expressed as mean $\pm$ standard deviation).

Group 3. Diabetic neuropathy The mean number of nuclei per muscle fibre in diabetic patients was 3.63 ± 0.93 (range from 2.46 to 5.41) and in controls was 2.14 ± 0.25 (range from 1.94 to 2.56). This difference was statistically significant ($p < 0.001$). As it can be seen from Table 5.IV. and Fig. 5.2., there is only a slight overlap of values between the two groups. The observed variation in control values seems unlikely to reflect age differences since, after puberty, the number of nuclei per muscle fibre remains fairly constant (Vassilopoulos et al. 1976 c). In any case, this variation is minimal compared to the marked increase of nuclei per muscle fibre in patients with diabetic neuropathy. In the present study, no correlation could be detected between the

number of nuclei per muscle fibre and various clinical parameters of the diabetic patients (duration of illness, severity of diabetes etc.).

	Age	Sex	Number of nuclei	
			mean±S. D.	range
Controls	48.9	4M 4F	1.24±0.25	1.94±2.56
Diabetics	61.1	4M 4F	3.63±0.93	2.46±5.41

Table 5.IV. Number of nuclei per muscle fibre in controls and patients with diabetic neuropathy.

The increase observed in the diabetic group corresponded to an increase in the number of myonuclei (Plate 5.B), identified at the ultrastructural level (Plate 5.B).

Group 4. Myotonic dystrophy. The mean number of nuclei per muscle fibre in the patients with myotonic dystrophy (3.73±0.81) is considerably higher ($p < 0.001$) than that of the controls (2.24±0.17). As it can be seen from Table 5.V. and Fig. 5.3., there is only a very slight overlap of the individual values between the two groups.

The observed increased number of nuclei per muscle fibre in the myotonic group did not correlate with an individual's age, sex, duration of illness or any other parameter studied.

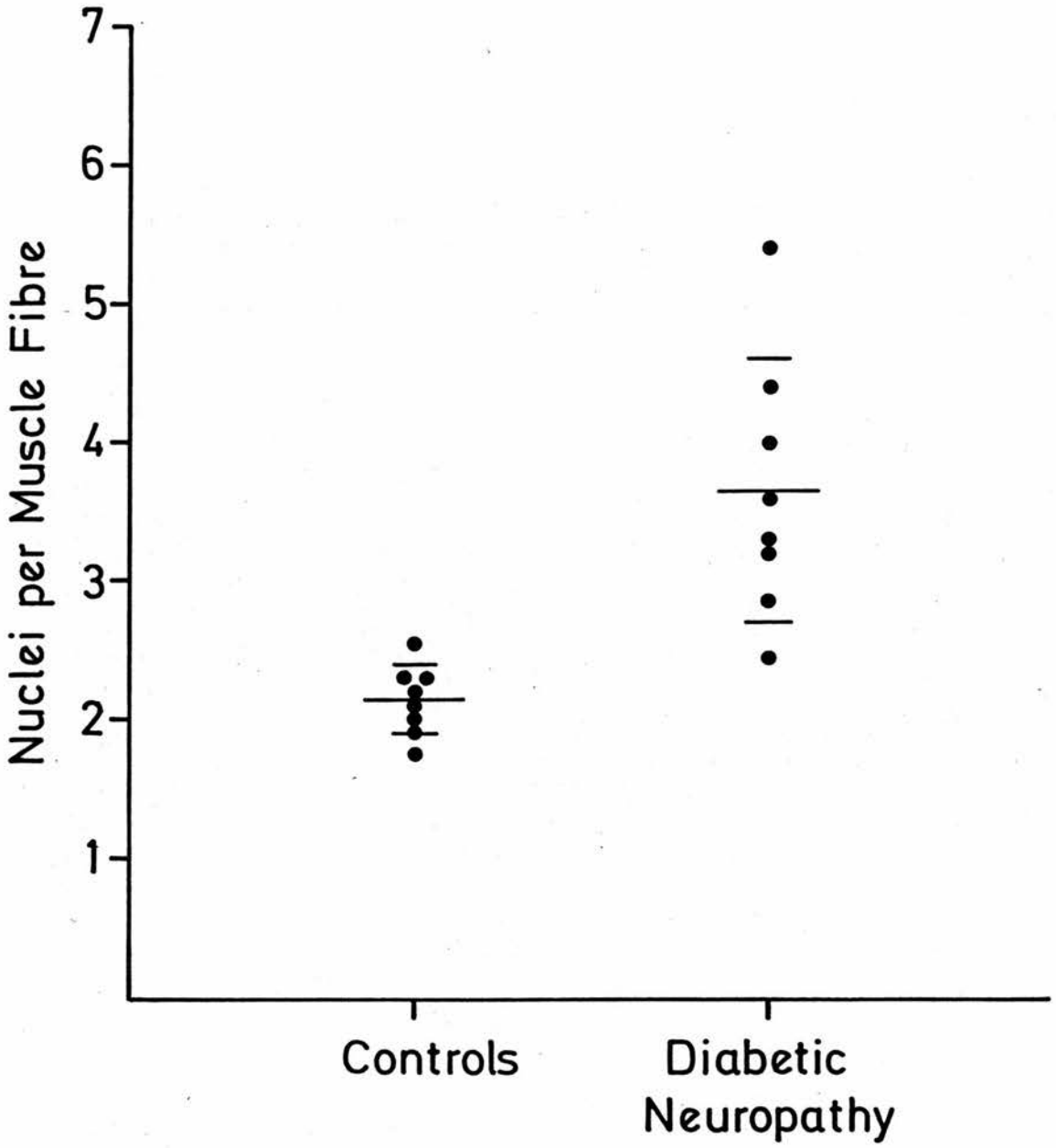


Figure 5.2. Mean number of nuclei per muscle fibre in controls and patients with diabetic neuropathy.

	Identification	Age (years)	Sex	Nuclei per fibre	
				Sarcolemmal	Central
Controls	A. R.	30	F	2.19	0.04
	R. M.	55	M	2.37	-
	I. A.	43	F	1.94	0.06
	J. M.	32	M	2.47	-
	W. M.	49	M	2.14	0.03
	J. W.	27	F	2.32	0.02
	E. L.	26	F	2.27	-
	J. C.	50	M	2.47	-
Myotonic dystrophy	M. S.	33	M	4.94	1.33
	S. L.	35	F	2.42	0.02
	M. R.	39	F	2.87	1.07
	A. B.	37	M	4.37	-
	M. Q.	37	F	4.21	-
	J. C.	48	M	3.85	1.73
	R. W.	43	F	4.11	0.01
	B. B.	41	F	3.68	0.40

Table 5. V. Number of sarcolemmal and central nuclei per muscle fibre in biopsies from Controls and patients with Myotonic Dystrophy.

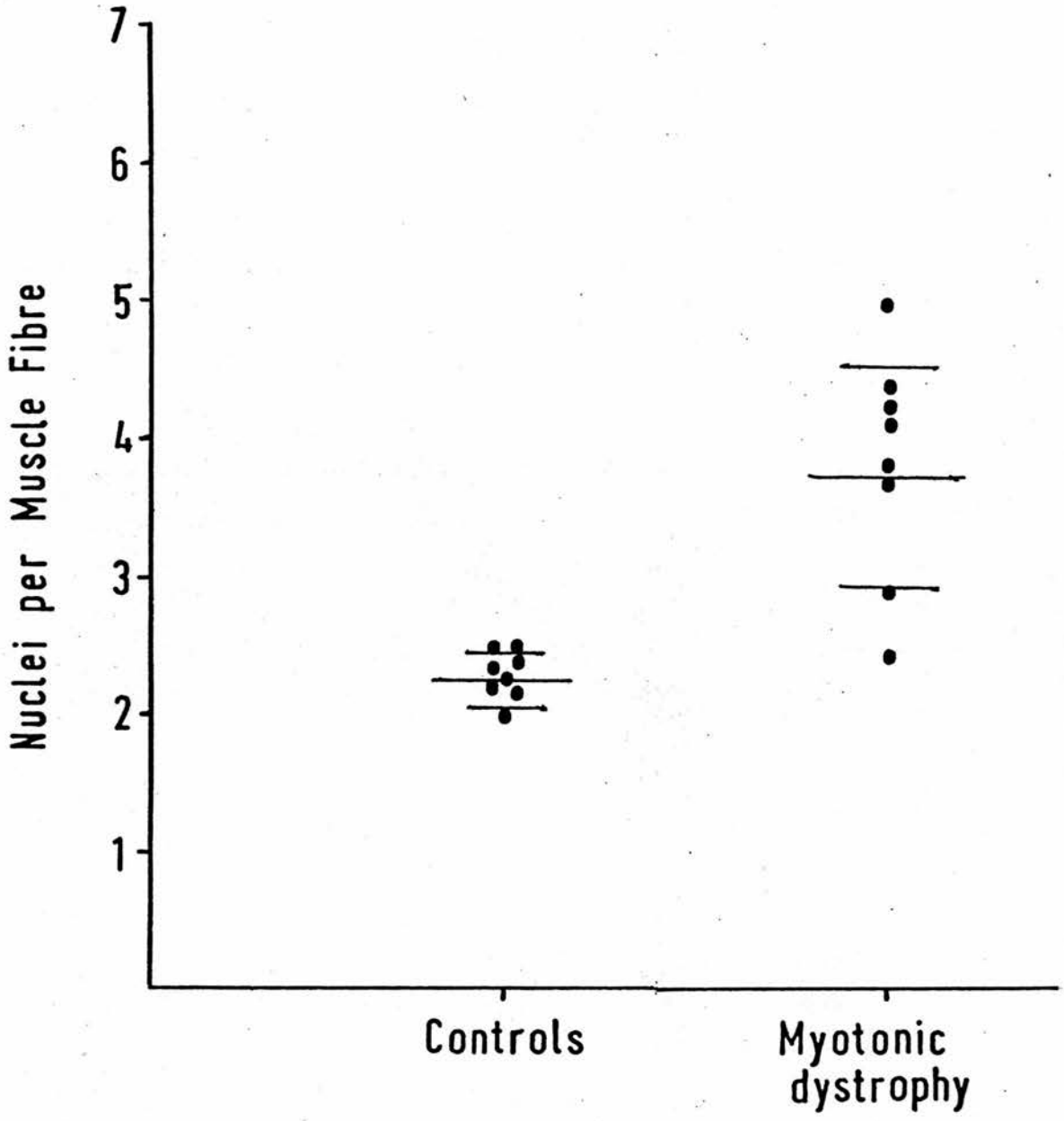


Figure 5.3. Mean number of nuclei per muscle fibre in controls and patients with Myotonic Dystrophy.

IV. Discussion

The nuclear changes observed in this study were of two different types i.e. an increase in muscle nuclear size (Duchenne muscular dystrophy) and an increase in the number of nuclei per muscle fibre (neuropathies, myotonic dystrophy).

Altered nuclear size has been observed in a number of human diseases. Guimarães (1971) found significantly increased nuclear volumes in the cirrhotic liver, but this enlargement was not correlated with the functional activity of the hepatocytes. Heiberg (1957) described quantitative nuclear changes in cancer cells which he regarded as fundamental not only as a diagnostic aid, but also indicative of the prognosis of various malignant tumours.

Alterations in nuclear size have also been detected in a number of experimental conditions. Bandaranayake, (1974) observing alterations in the size of the hypothalamic neurone nuclei under certain experimental conditions, (dehydration, water loading, lactation etc.) related this to the functional state of the organ. Alfert and his colleagues (1955) found enlarged thyroid epithelium nuclei and, in addition, they concluded that this increase was due to increased amounts of non-histone proteins since the DNA and histone content remained unchanged.

Various attempts have been made to correlate the shape of muscle cell nuclei with various conditions such as the degree of muscle contraction, altered ionic environment

and cellular hypertrophy (Doljanski 1960, Davies and Spencer 1962, Goss 1964, Franke and Schinko 1969). The reasons for the increase in size of muscle fibre nuclei observed in this study in Duchenne muscular dystrophy are not immediately apparent. This change in nuclear size is unlikely to be a direct result of the primary gene defect and it is probably a reflection of an altered nucleo-cytoplasmic relationship.

Much information has recently been gained from nuclear transplantation experiments in amphibia (Gurdon 1968, 1970). These studies showed that when nuclei from fully differentiated cells (for example, adult Xenopus brain) are injected into maturing oocytes they suffer a pronounced enlargement and start dividing. This enlargement seems to be the result of a specific uptake of proteins from the cytoplasm (Arms 1968, Merriam 1969, Goldstein 1974) and although the exact nature of this mechanism is not fully understood, the influx of proteins into the nucleus seems to be related to alterations in the genome activity. The increase in size of the transplanted nuclei, and the uptake of cytoplasmic proteins, is accompanied by changes in the chromatin, which loses "heterochromatic clumped" structure and takes up a more dispersed form. This suggests that the uptake of proteins may represent changes in the organization of chromatin, so that it reprogrammes its state of gene expression to fit its new environment (Lewin 1974). Gurdon and Woodland (1970)

suggested that acidic nuclear proteins play an important role in these changes. It seems likely that the cytoplasm contains specific information which elicits the appropriate response from any nucleus placed in it, and so nuclei establish activities characteristic of their surrounding cytoplasm. This provides a possible explanation of the observed enlargement of the muscle fibre nucleus in Duchenne muscular dystrophy. In addition to DNA, the nucleus contains appreciable quantities of histone and acidic (non-histone) nuclear proteins. Current concepts of the regulation of gene expression consider that histones repress genes whereas acidic nuclear proteins selectively derepress genes (see Chapter 1, pages 15-18). A modification in the amount of the nuclear components (and acidic nuclear proteins in particular) in response to increased functional demands of the cytoplasm may explain the observed increased nuclear size in the dystrophic muscle.

Changes in nuclear number have also been reported. An increased number has been noted previously in diabetic neuropathy (Locke et al. 1963, Bloodworth and Epstein 1967, Malene and Stroia 1974), but little significance was attached to this finding at the time. Also nuclear changes such as an increase in the number of sarcolemmal (Adams 1974) and central nuclei (Dubowitz and Brooke 1973, Adams 1974) have been observed in myotonic dystrophy.

Our results provide a quantitative estimate of the

increase in the number of sarcolemmal nuclei in muscle from patients suffering from diabetic neuropathy, other chronic neuropathies and myotonic dystrophy. Electron microscopic studies have shown that this increase in nuclei may be attributed to an increase in the number of myonuclei rather than satellite cell or connective tissue cell nuclei.

It is generally accepted that in human adult muscle the myonuclei do not divide. However, the origin of extra nuclei is suggested from animal experiments where labelling studies have shown that the myonuclei do not divide in the adult rat (MacConnachie et al. 1964, Moss and Leblond 1971), but that the extra nuclei needed during normal growth arise from satellite cell division; one daughter nucleus is incorporated into the muscle fibre forming a myonucleus and the second daughter nucleus goes to form a new satellite cell (Enesco and Puddy 1964).

Satellite cells have been implicated in a variety of physiologic and pathologic processes involving striated muscle. These cells may be involved in muscle development (Shafiq et al. 1968, Church 1969, Moss and Leblond 1971), response to injury and regeneration (Church et al. 1966, Reznik 1969) and reaction to muscular dystrophy, polymyositis and denervation (De Recondo et al. 1966, Shafiq et al. 1957, Hess and Rosner 1970), but their precise role has not yet been established. They have the same embryological origin as the muscle cell (Church 1970) suggesting that they possibly

have a specific role in muscle fibre function, but they do not fuse to form myotubes. The presence of centrioles in the satellite cell cytoplasm and their absence within the multinucleated muscle cell cytoplasm (Muir 1970) suggests that their role is related to mitosis and the formation of new nuclei.

The only other syncytial tissue of the human body, the syncytiotrophoblast of the placenta may provide a functional analogy. In this case, the function of the cytotrophoblast which is a mononucleated cell with certain anatomical similarities to the muscle satellite cell, is to contribute nuclei to the syncytiotrophoblast (Enders 1965). It seems reasonable to assume that in humans, satellite cells operate in the same manner as is suggested by Enesco and Puddy (1964) in animal muscle.

The increased number of sarcolemmal nuclei observed in the chronic neurogenic atrophies, in diabetic neuropathy and in myotonic dystrophy, may be due to an increase in mitotic activity of the satellite cells. It seems reasonable to suggest that the extra nuclei observed in denervated muscle come from satellite cell division. However, the reason for the differing reaction of the satellite cells to neuropathic and myopathic processes needs further study.

Support for this suggestion comes from the observation of Farkas et al. (1974) that in the muscle of myotonic children the increase in the centrally placed nuclei is paralleled by an

increase in the number and size of the satellite cells. The authors considered this finding more important than the centralization of the nuclei and pointed out that an increase in satellite cell number as in young myotonics is not present in other congenital myopathies, but is less pronounced than that observed by Van Haelst (1970) in a child with Werdnig-Hoffmann's disease.

Whether the observed increase in the number of nuclei per muscle fibre in chronic denervated muscle, in diabetic neuropathy and myotonic dystrophy reflects the same underlying pathogenic mechanism or is the end result of different processes cannot at present be determined. However, the differences in muscle nuclear size between Duchenne muscular dystrophy and spinal muscular atrophies indicates a fundamental difference between neuropathies and myopathies. This difference in the response to the disease process between these two major groups of diseases, is emphasized by the increase in the number of nuclei per muscle fibre in neuropathies, which is not present in the muscular dystrophies.

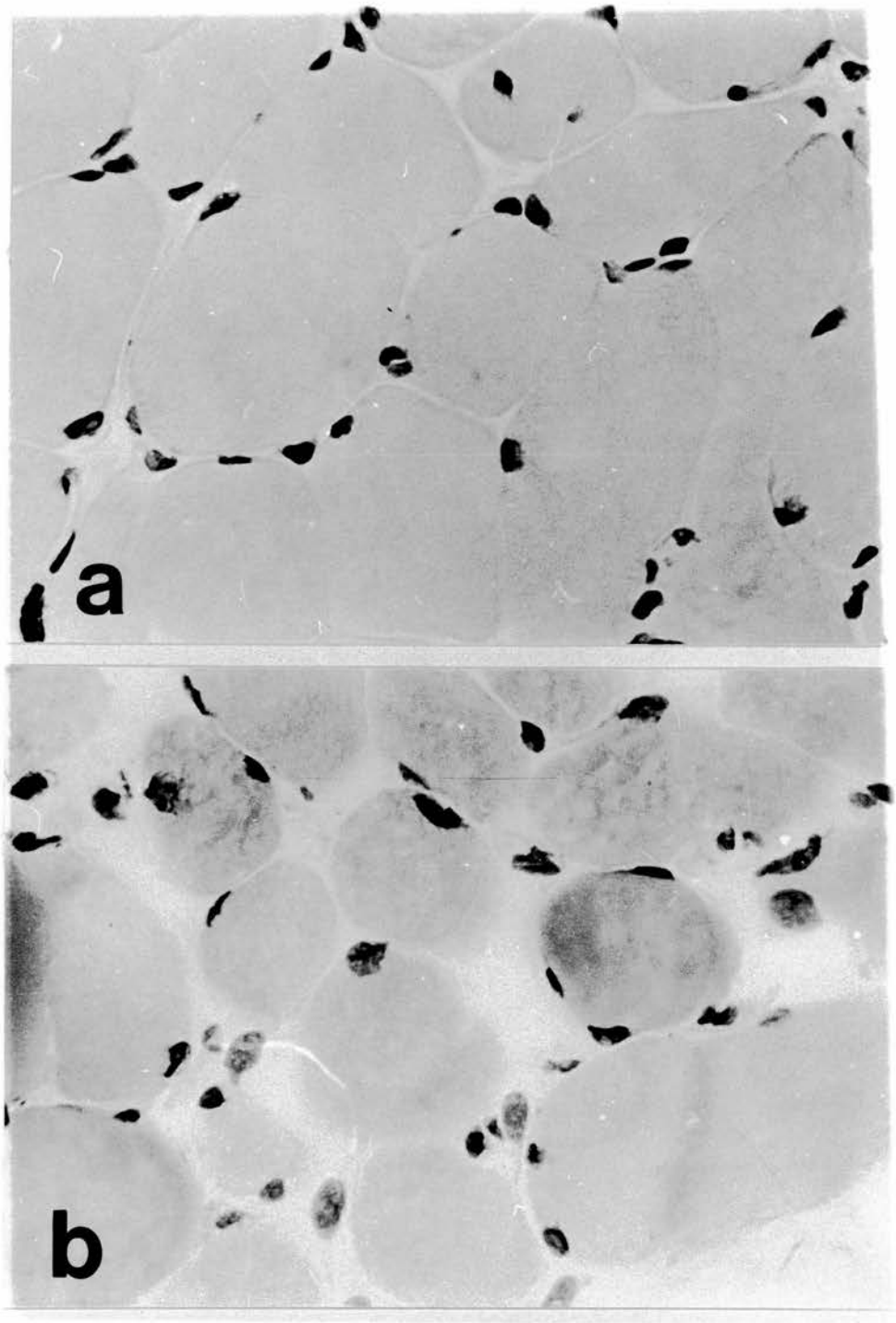


Plate 5. A. Transverse section of muscle in spinal muscular atrophy (a) and Duchenne muscular dystrophy (b). Cryostat sections stained with haematoxylin and eosin (x 700).

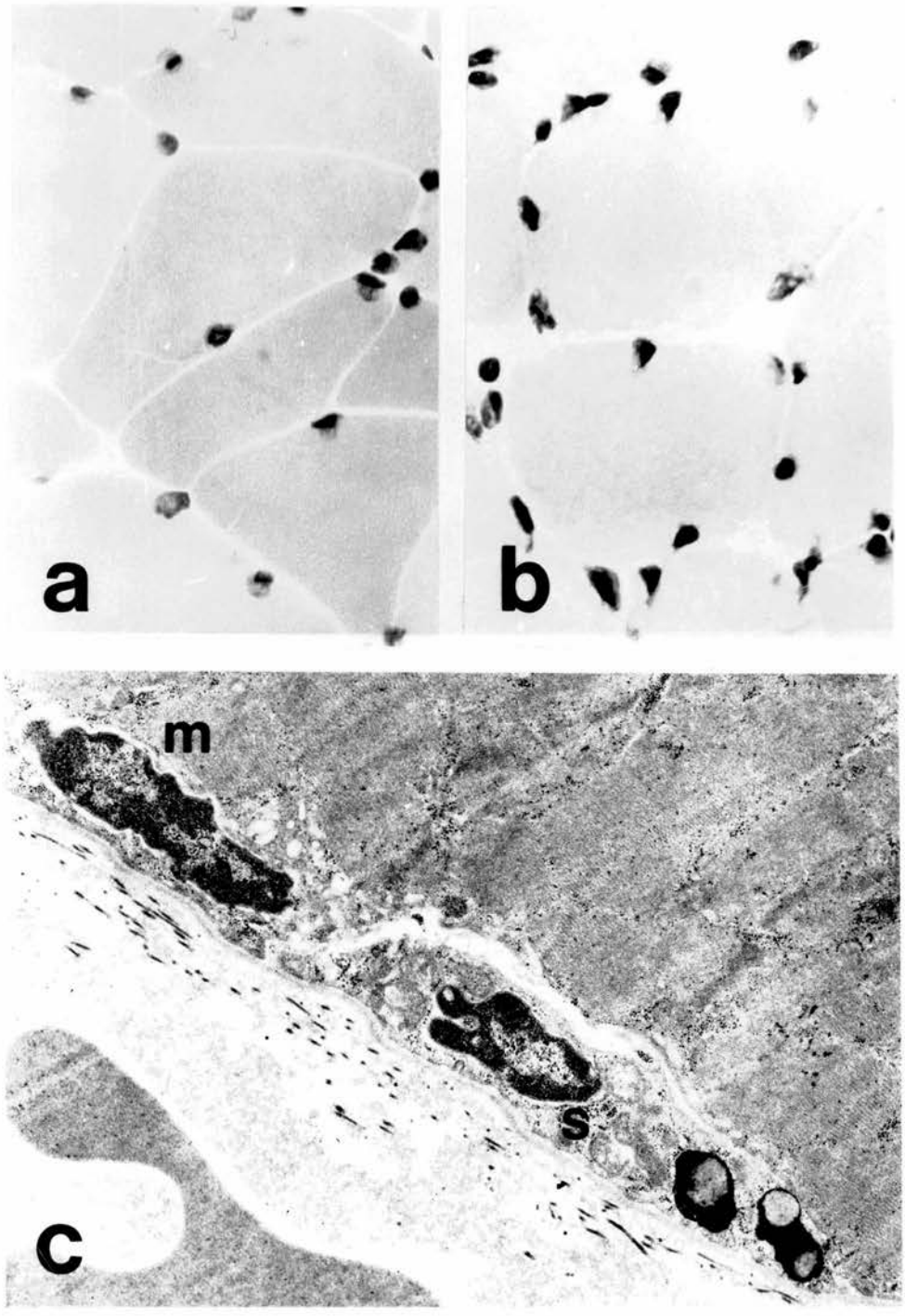


Plate 5.B. Transversely sectioned muscle fibres from a normal adult (a) and a patient with diabetic neuropathy (b). Frozen sections stained with haematoxylin and eosin (x 700). Electron micrograph (c) from transversely sectioned muscle fibres showing a myonucleus (m) and a satellite cell nucleus (s). Lead citrate and uranyl acetate (x 16,000).

CHAPTER 6

The fetal development of the human cervical spine and cord

I. Introduction

II. Material and Methods

i Material

ii Methods

III. Results

IV. Discussion

CHAPTER SIX

I. Introduction

Many common neurological syndromes are believed to associated with anatomical modifications of the cervical spine. The most common of these syndromes is the so-called "cervical spondylotic myelopathy", in which a compression of a normally developed spinal cord by a congenitally narrow vertebral canal is believed to be the main pathogenic factor (Wolf et al. 1956, Payne and Spillane 1957, Burrows 1963, Bradley et al. 1968). This discrepancy in size between vertebral canal and spinal cord is suggestive of differing

patterns of development. On the contrary, in spinocerebellar ataxia (a hereditary disorder of the nervous system) the developmental pattern of these two organs seems to be similar, since the existing atrophic spinal cord (Brain and Walton 1969) is associated with a narrow vertebral canal (Vassilopoulos and Spengos 1975). The relative size of the vertebral canal and spinal cord seems to be of considerable importance in the understanding of the pathogenic mechanisms of some conditions such as syringomyelia, hydromyelia, spina bifida and other "dysraphic conditions".

In the human adult, under normal conditions, the size and the proportion of the various organs is genetically determined (Bertalanffy 1960). Changes in the proportion of the organs are considered to be the result of disturbed relative growth, that is the ratio of growth rates of the various parts of the body.

The present study, of the development of the vertebral canal and spinal cord during fetal life, was undertaken in the hope that the elucidation of the growth pattern of these organs would prove helpful in a better understanding of the relationship between vertebral canal and spinal cord in health and disease. The results of this study have been already published (Vassilopoulos 1976).

II. Material and Methods

i Material

The material for this study consisted of 66 normal fetuses at various gestational ages (6 to 26 weeks), obtained from prostaglandin induced abortions for social reasons. The material was grouped according to the gestational period and the number of fetuses in each one of these groups is shown in Table 6.I. The gestational period was estimated by each of three methods i.e. heel-toe and crown-rump length measurements, as well as menstrual age (see Chapter 1). When there was a discrepancy among these estimations, the heel-toe and crown-rump lengths were considered as characteristics of the gestational age.

ii Methods

The sixth cervical vertebra was removed and the anterio-posterior as well as the transverse diameters of the vertebral canal and spinal cord (Fig. 6.1) were measured under a stereo microscope (X16 magnification) with the aid of a calibrated eye piece.

The vertebral canal and the spinal cord have different shapes, so in order to obtain a morphometric comparison of these two organs, the areas were estimated on the assumption that the vertebral canal is approximately triangular and the spinal cord approximately elliptical.

The cross-sectional areas for the canal and cord were plotted against the gestational age of the fetus and a computer

programme was used in order to derive the best fitting curve through the points (Fig. 6.2).

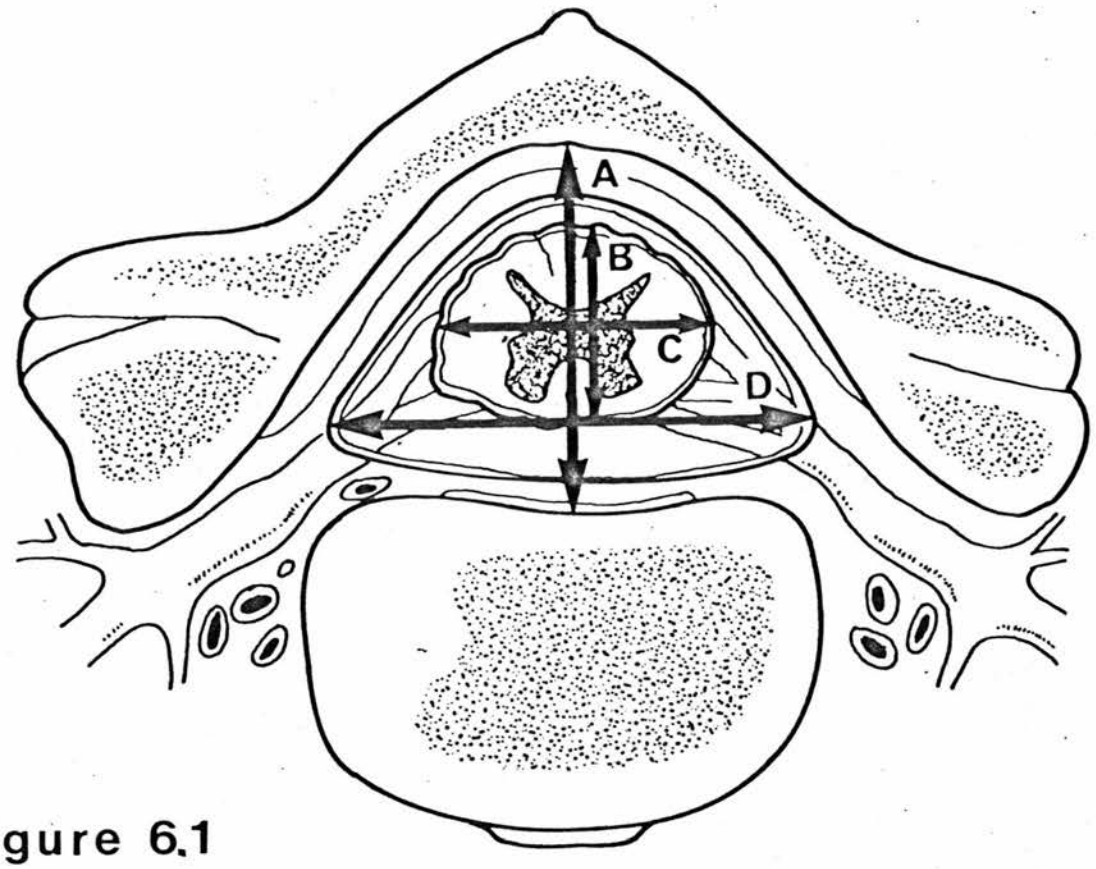


Figure 6.1

In the growth of an organism as a whole, time and body size are the two variables under consideration. Relative growth concerns the ratio of the rates of growth between several components of the organism and this ratio can be well expressed by the "allometric equation" (Huxley 1932, Bertalanffy 1960).

$$Y = bX^{\alpha} \quad \text{or} \quad \log Y = \log b + \alpha \log X$$

(In this equation α is the slope of the allometric line and if $\alpha > 1$, Y grows faster than X or shows positive allometry. If $\alpha = 1$ both grow at the same rate or isometrically and

if $\alpha < 1$ the relative increase of Y is smaller than that of X or Y shows a negative allometry (Bertalanffy 1960).

Since the vertebral canal and the spinal cord are essentially parts of the developing body, it was considered that this equation would provide a better representation of the relative growth of these organs. A computer programme was used to estimate the "coefficient of allometry" (α).

III. Results

The results of this study are shown in Tables 6.I and 6.II. It can be seen from the Table I that both the anterio-posterior as well as the transverse diameters of the vertebral canal and the spinal cord exhibit the same rate of growth. This development can be expressed diagramatically as a continuous process (Fig. 6.2) without any sudden discontinuity. Computer analysis suggested that the best equations for expressing the growth rates were:

Vertebral canal $Y = (-361.3)\ X + (28.9)\ X^2 - (0.5)\ X^3 + 1501$

Spinal cord $Y = (-318)\ X + (26.5)\ X^2 - (0.5)\ X^3 + 1267$

Gestation (weeks)	No.	Vertebral Canal		Spinal Cord	
		Anterio-posterior	Transverse	Anterio-posterior	Transverse
6-8	6	1,70±0,26	2,51±0,30	1,20±0,18	1,85±0,20
9-11	9	2,13±0,17	3,21±0,33	1,47±0,15	2,26±0,18
12-14	17	2,81±0,36	4,19±0,48	2,06±0,31	3,08±0,45
15-17	14	3,75±0,35	5,16±0,44	2,66±0,34	3,50±0,10
18-20	10	4,54±0,45	6,52±0,84	3,23±0,42	4,57±0,54
21-23	7	5,68±0,25	7,42±0,26	3,90±0,25	5,31±0,26
24-26	3	6,86±0,05	8,50±0,43	4,59±0,05	5,93±0,15

Table 6.I. Anterio-posterior and transverse diameters of the Vertebral Canal and Spinal Cord. (mean ± one standard deviation)

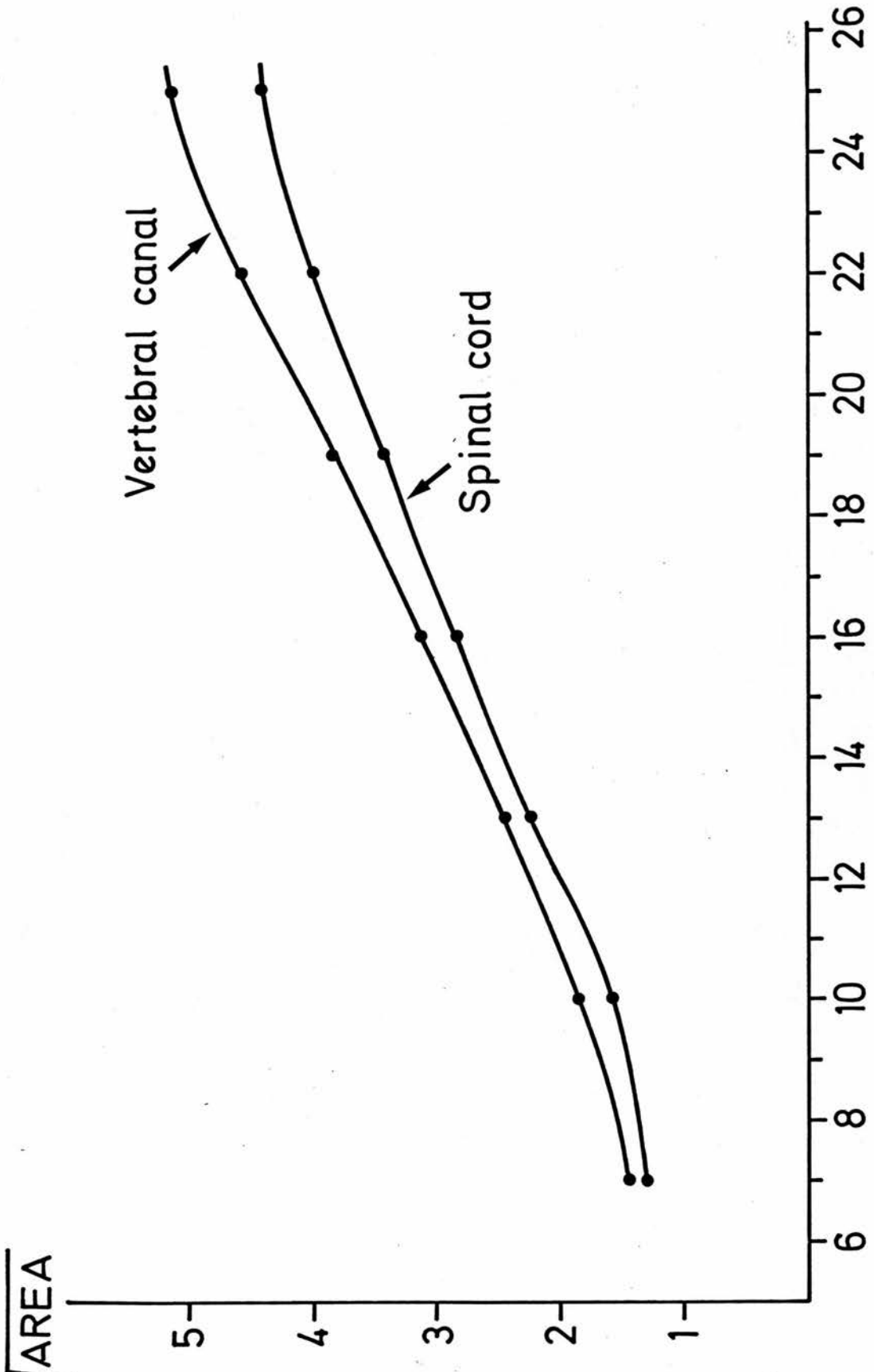


Figure 6.2. Developmental pattern of the Vertebral canal and Spinal cord. (The values represent the square root of the cross-sectional areas - mm).

The similarity of these two equations suggests that these two parts of the body exhibit the same developmental process during their growth in fetal life.

In addition, the computer analysis for "allometry" gave the following results:

$$\text{Vertebral canal} \quad Y = (2.36)\, X^{0.5544}$$

$$\text{Spinal Cord} \quad Y = (2.11)\, X^{0.5543}$$

Gestation (weeks)	No.	Vertebral Canal	Spinal Cord
6-8	6	$2,16 \pm 0,55$	$1,76 \pm 0,45$
9-11	9	$3,41 \pm 0,35$	$2,58 \pm 0,28$
12-14	17	$5,99 \pm 1,29$	$5,09 \pm 1,36$
15-17	14	$9,78 \pm 1,43$	$8,06 \pm 1,58$
18-20	10	$14,95 \pm 3,23$	$11,72 \pm 2,76$
21-23	7	$21,14 \pm 1,24$	$15,88 \pm 0,98$
24-26	3	$26,50 \pm 1,46$	$19,22 \pm 1,45$

Table 6.II. Cross-sectional areas of the Vertebral Canal and Spinal Cord (mean $\pm$ one standard deviation)

IV. Discussion

Many measurements of the vertebral canal have been reported since the work of Elsberg and Dyke (1934) who studied

the interpendicular distance of the vertebrae as a diagnostic criterion for the detection of intravertebral tumours. In view of the diagnostic value, a number of authors estimated normal values for the various levels of the vertebral canal in infants and children (French et al. 1942, Anderson et al. 1953, Simril et al. 1955, Schwarz 1956, Hinck et al. 1965).

In spite of this amount of research, the developmental pattern of the vertebral canal remained unclear probably due to the widely differing values reported by these authors resulting from differing methodological techniques. Schwarz (1956) suggested that the growth of the vertebral canal is continuous and this is in disagreement with the "stair-step" manner of growth favoured by Simril et al. (1955). As far as the spinal cord is concerned, values for its size during development, have not yet been established, although adult values have been repeatedly reported.

In this study, the results indicate that the vertebral canal and the spinal cord exhibit the same continuous rate of growth, and this is similar for the two dimensions studied (i. e. anterio-posterior and transverse). This continuous pattern of growth supports Schwarz's (1956) hypothesis on the development of the vertebral canal.

Morphogenetic changes in a growing animal chiefly take place by relative growth, that is certain components increase at a higher or lower rate than others, or, as also can be said, growth rate is different in different spatial dimensions.

The proportions of adult human body are established in the way that its various parts have different, well-programmed, spatial and temporal growth.

The two conclusions coming from the equations of allometry are: Firstly, the similarity of these equations suggests the same pattern of growth for both the vertebral canal and the spinal cord although the spinal cord development seems slightly slower. This pattern of growth explains the finding that in spinocerebellar ataxia the smaller spinal cord (Brain 1969) is associated with a smaller vertebral canal (Vassilopoulos and Spengos 1975), however, the problem of the discrepancy in size between canal and cord in cervical spondylotic myelopathy remains unanswered. Secondly, both the vertebral canal and the spinal cord develop considerably slower than the body as a whole, as indicated by the coefficients of allometry (0.5544 and 0.5543 respectively). This rate of growth is also considerably slower than other organs. It has been reported that in human heart, kidneys, spleen and musculature have approximately the same rate of growth which is higher than that calculated for the vertebral canal and spinal cord (Linzbach 1955).

It should be noted that the growth of human brain is also slower than that of various organs (musculature, spleen, kidneys) (Linzbach 1955). Dubois (1930) reported that in a series of mammals the coefficient of allometry for the brain was $\alpha = 5/9$ (0.556) which is similar to the values obtained

for the vertebral canal and the spinal cord. So, it seems reasonable to suggest that the growth pattern of the spine is different from that of other organs but is similar to that of the brain, a finding indicating a similarity in the genetically determined growth pattern of the various parts of the nervous system.

The postnatal development of the human brain is well documented and the same is true for the vertebral canal, but the pattern of the postnatal development of the spinal cord is still unclear. Knowledge of the complete growth pattern would allow a better approach to the determination of developmental alterations in the cervical spine and a better understanding of the pathogenesis of these syndromes.

CONCLUSIONS

CONCLUSIONS

The studies undertaken during this project have concentrated on the pattern of nuclear changes in spinal motor neurones and in muscle fibres during normal development and in some neuromuscular diseases. Fetal spinal cord (lumbar region) and fetal muscle (gastrocnemius) was used as well as muscle biopsies from children and adults with various neuromuscular disorders. The histochemical stains used throughout the study were identical to standard techniques used to stain adult tissues. Some factors which might influence the results and therefore the validity of the interpretations will be briefly discussed.

The first factor is the presumed normality of the fetal material and the control biopsy material used. The fetuses were presumed normal because there was no apparent abnormality on gross examination and no family history suggesting that possibility. The biopsy material was assumed to be normal if there was no apparent histological or histochemical abnormality based on our laboratory's extensive experience.

The second factor concerns the stains used. A difficulty arose when Toluidine blue (at pH 9.0) was used for muscle. This was because Toluidine blue stains the muscle cytoplasm so intensively that the nucleus is difficult to distinguish.

A third factor was the difficulty in recognizing spinal motor neurones in very young fetuses (of gestational age less than 9-10 weeks).

Some other factors concern the biopsy material used. For example it was very difficult to obtain control muscle from the same muscle as the abnormal muscle and of a comparable age to the disease groups studied. The muscle biopsied in the majority of the cases was the quadriceps femoris, but the vastus lateralis and the gastrocnemius were also used.

After allowing for these points, the results of the motor neurone study suggested that the 12th to 14th week period is critical for differentiation of anterior horn cells. At this

period an increase in acidic nuclear proteins and arginine-rich histones was observed and this was accompanied by an increase in neurone somal, nuclear and nucleolar size. This study provided a normal pattern for the development of the nucleus of the motor neurones for comparison with data from abnormal material.

The study of the muscle fibre revealed no changes in the size or in the composition of its nucleus during fetal development. However, in normal postnatal growth, the increase in muscle fibre size is paralleled by a similar increase in the number of myonuclei and these changes seem to keep the nucleo-cytoplasmic ratio within certain limits.

The study of diseased muscle showed an enlargement of the muscle nucleus in biopsies from children with Duchenne muscular dystrophy in at least four out of eight fetuses at risk and in dystrophic muscle under in vitro conditions. This finding seems to reflect a nuclear response to the dystrophic process.

An increase in the number of nuclei per cross-sectioned muscle fibre was detected in chronic neurogenic atrophies, in diabetic neuropathy and in myotonic dystrophy. It was suggested that the extra nuclei come from satellite cell division. This finding of an increased number of nuclei was not present in muscular dystrophies. This indicates a fundamental difference in the response of the muscle fibre nucleus to the neurogenic and myopathic processes.

Finally, the results from the study of the canal and spinal cord growth during normal fetal development suggested that these two organs of the developing human have the same developmental pattern. Therrate of their growth is lower than that of the body as a whole, but similar to that reported for the brain. This may indicate a similarity in the genetic factors determining growth for all the various parts of the nervous system.

The process of cell differentiation is very complex and not fully understood, so these results should be interpreted with caution. When we understand the mechanisms which control protein synthesis, better, we should be able to get a clearer picture of neuronal and muscle fibre differentiation and how they grow during both fetal and postnatal life.

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A P P E N D I X

1. Motor neurone measurements
2. Muscle measurements
3. Solutions and source of chemicals

SPINAL MOTOR NEURONE

Fetus no.	Gestational age (weeks)	DNA	Histones			Acidic nuclear proteins	Neurone vol. (μm^3)	Nuclear vol. (μm^3)
			Total	Lysine-rich	Arginine-rich			
3	26	0.70	1.12	0.88	0.24	1.03	9980	850
10	21	0.62	1.10	0.93	0.17	1.17	8060	685
11	13	0.78	1.75	0.98	0.77	1.35	2162	174
12	20	0.71	1.61	0.99	0.17	1.23	5135	548
14	14	0.59	1.77	0.94	0.83	1.00	6575	608
15	17	0.52	1.55	0.90	0.65	1.01	5506	449
16	10	0.65	0.95	0.82	0.13	0.88	5895	556
12	15+	0.72	1.15	0.91	0.24	0.98	2896	185
20	15+	0.61	1.24	1.03	0.21	1.23	7094	660
21	19	0.60	1.12	0.92	0.20	1.03	6950	635
22	14	0.70	1.03	0.89	0.14	1.35	6666	409
24	17	0.75	1.39	1.09	0.30	1.24	4229	163
25	11	0.63	1.35	1.06	0.29	1.20	4319	213
26	12	0.54	1.38	1.18	0.20	1.35	2412	245
27	19	0.58	1.05	0.89	0.16	0.92	8483	432

SPINAL MOTOR NEURONE (continued)

Fetus no.	Gestational age (weeks)	DNA	Histones			Acidic nuclear proteins	Neurone vol. (μm^3)	Nuclear vol. (μm^3)
			Total	Lysine-rich	Arginine-rich			
28	17	0.68	0.83	0.79	0.04	0.85	8210	658
29	16	0.62	1.59	0.91	0.64	1.20	6105	374
30	11	0.58	0.86	0.79	0.07	1.01	4196	439
31	6	0.64	1.16	0.87	0.29	0.87	2389	203
32	7	0.55	1.06	0.89	0.17	0.97	1739	171
37	24	0.59	1.06	0.93	0.13	1.09	10159	930
38	10	0.75	0.83	0.80	0.03	1.10	3285	264
39	18	0.57	1.14	1.09	0.05	0.99	4937	292
40	22	0.60	1.14	0.91	0.23	1.16	7294	531
41	6	0.68	1.12	0.92	0.20	0.94	3700	207
42	11	0.71	1.31	1.08	0.23	1.03	2203	222
43	18	0.69	1.11	0.98	0.13	1.24	8958	940
51	22	0.70	1.12	1.06	0.06	1.01	9816	750
54	16	0.69	1.06	0.88	0.18	1.17	5065	451
55	18	0.62	1.05	0.87	0.18	0.97	5930	620

SPINAL MOTOR NEURONE (continued)

Fetus no.	Gestational age (weeks)	DNA	Histones			Acidic nuclear proteins	Neurone vol. (μm^3)	Nuclear vol. (μm^3)
			Total	Lysine-rich	Arginine-rich			
56	12	0.63	1.15	0.96	0.19	1.05	8470	634
57	12	0.68	1.16	0.93	0.23	1.10	8396	480
58	14	0.69	1.35	1.08	0.27	1.16	5976	850
59	12	0.72	1.43	0.97	0.46	1.22	3729	426
60	12	0.55	1.01	0.88	0.13	1.13	4238	514
63	23	0.65	1.00	0.82	0.18	1.05	7100	801
64	8	0.59	1.07	0.91	0.16	1.04	3150	192
65	13	0.58	1.41	1.06	0.35	1.37	6832	701
66	25	0.63	1.08	0.97	0.11	1.08	9360	730
67	21	0.58	1.08	0.86	0.22	0.99	7385	624
68	24	0.69	1.10	1.01	0.09	1.08	9573	810
69	15	0.59	1.28	0.93	0.35	1.06	4013	198

MUSCLE

Fetus no.	Gestational age (weeks)	DNA	Histones			Acidic nuclear proteins	Nuclear area (μm^2)
			Total	Lysine-rich	Arginine-rich		
3	26	0.78	0.68	0.61	0.07	1.09	27.1
10	21	0.73	0.85	0.80	0.05	1.06	24.3
11	13	0.69	0.95	0.83	0.12	1.27	26.0
12	20	0.71	0.78	0.71	0.07	1.17	25.3
14	14	0.72	0.73	0.64	0.09	1.09	30.9
15	17	0.68	0.68	0.61	0.07	1.07	24.2
16	10	0.81	0.88	0.75	0.13	1.19	29.4
17	11	0.73	0.64	0.56	0.08	1.12	25.2
19	15+	0.69	0.79	0.65	0.14	1.18	30.0
20	15+	0.69	0.80	0.71	0.09	1.22	32.3
21	19	0.69	0.65	0.61	0.04	1.21	26.1
22	14	0.71	0.96	0.80	0.16	1.17	19.2
24	17	0.71	0.95	0.82	0.13	1.06	19.3
25	11	0.62	0.62	0.53	0.09	1.25	26.6
26	12	0.79	0.68	0.62	0.08	1.28	23.1
27	19	0.69	0.81	0.69	0.12	0.20	24.7

MUSCLE (continued)

Fetus no.	Gestational age (weeks)	DNA	Histones			Acidic nuclear proteins	Nuclear area (μm^2)
			Total	Lysine-rich	Arginine-rich		
28	17	0.72	0.69	0.60	0.09	1.11	22.3
29	16	0.78	0.66	0.51	0.15	1.37	30.9
30	11	0.68	0.84	0.80	0.04	1.33	21.9
31	6	0.63	0.63	0.60	0.03	1.11	21.5
32	7	0.79	0.52	0.46	0.06	1.33	22.4
37	24	0.73	0.93	0.82	0.09	1.11	22.7
38	10	0.79	0.76	0.70	0.06	1.10	22.6
39	18	0.70	0.70	0.61	0.09	1.07	31.0
40	22	0.69	0.67	0.60	0.07	1.32	29.8
41	6	0.68	0.88	0.70	0.18	1.16	23.0
42	11	0.69	0.92	0.77	0.15	1.28	28.4
43	18	0.78	0.83	0.73	0.10	1.36	27.4
51	22	0.67	0.93	0.79	0.14	1.13	21.9
54	16	0.69	0.84	0.71	0.13	1.23	30.2
55	18	0.74	0.84	0.81	0.03	1.11	25.6
56	12	0.65	0.78	0.73	0.05	1.10	27.1

MUSCLE (continued)

Fetus no.	Gestational age (weeks)	DNA	Histones			Acidic nuclear proteins	Nuclear area (μm^2)
			Total	Lysine-rich	Arginine-rich		
57	12	0.67	0.82	0.71	0.11	1.12	27.2
58	14	0.72	0.63	0.55	0.08	1.16	23.7
59	12	0.69	0.71	0.62	0.09	1.26	29.7
60	12	0.73	0.74	0.61	0.13	1.11	24.9
63	23	0.71	0.90	0.78	0.12	1.19	25.4
64	8	0.71	0.80	0.72	0.08	1.17	24.8
65	13	0.70	0.71	0.65	0.06	1.17	23.6
66	25	0.69	0.75	0.68	0.07	1.27	23.8
67	21	0.71	0.68	0.62	0.06	1.18	25.9
68	24	0.69	0.81	0.70	0.11	1.16	29.2
69	15	0.79	0.74	0.70	0.04	1.20	-

SOLUTIONS and SOURCE of CHEMICALS

Feulgen reaction for DNA

Schiff's reagent — BDH Chemicals Ltd.

Acetic-Ethanol

glacial acetic acid 10 ml.

absolute ethyl alcohol 30 ml.

SO_2-water

1N hydrochloric acid 25 ml.

0.5% aqueous solution of potassium metabisulphite 50 ml.

Fast Green reaction for Histones

Neutral Formalin

37-41% Formaldehyde solution (BDH) 10 ml.

Distilled water 90 ml.

Calcium carbonate to excess

Trichloroacetic acid

5% in water

Fast Green FCF solution (Gurr Ltd.)

0.1% solution in distilled water

0.1 N NaOH (to pH 8.0-8.1)

Toluidine blue reaction for Acidic Nuclear Proteins

Neutral formalin

10% solution (As for Histones)

Trichloroacetic acid

5% in water

Toluidine blue - BDH Chemical Ltd.

0.5% solution in distilled water

(0.1 N NaOH to pH 9.0)

Phenanthrenquinone reaction for Arginine-rich Histones

Neutral formalin

10% solution (As for Histones)

Trichloroacetic acid

5% in water

Phenanthrenquinone solution

1 part 0.5 N NaOH
4 parts absolute alcohol
1 part fresh 1% Phenanthrenquinone (BDH) solution in Dimethyl-formamide (BDH)

PUBLICATIONS

PUBLICATIONS

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3. Vassilopoulos D. and Emery A. E. H. 'Muscle nuclear changes in fetuses at risk for Duchenne muscular dystrophy', Journal of Medical Genetics, (In Press).

4. Vassilopoulos D., Lumb E. M., Corrall R. J. M. and Emery A. E. H. 'Muscle karyometry in diabetic neuropathy', Zeitschrift für Neurologie (In Press).

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